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## The effect of morphine upon DNA methylation in ten regions of the rat brain

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#### ABSTRACT

Morphine is one of the most effective analgesics in medicine. However, its use is associated with the development of tolerance and dependence. Recent studies demonstrating epigenetic changes in the brain after exposure to opiates have provided insight into mechanisms possibly underlying addiction. In this study, we sought to identify epigenetic changes in ten regions of the rat brain following acute and chronic morphine exposure. We analyzed DNA methylation of six nuclear-encoded genes implicated in brain function (Bdnf, Comt, Il1b, Il6, Nr3c1, and Tnf) and three mitochondrially-encoded genes (Mtco1, Mtco2, and Mtco3), and measured global 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5 hmC) levels. We observed differential methylation of Bdnf and II6 in the pons, Nr3c1 in the cerebellum, and II1b in the hippocampus in response to acute morphine exposure (all P value < 0.05). Chronic exposure was associated with differential methylation of Bdnf and Comt in the pons, Nr3c1 in the hippocampus and II1b in the medulla oblongata (all P value < 0.05). Global 5mC levels significantly decreased in the superior colliculus following both acute and chronic morphine exposure, and increased in the hypothalamus following chronic exposure. Chronic exposure was also associated with significantly increased global 5hmC levels in the cerebral cortex, hippocampus, and hypothalamus, but significantly decreased in the midbrain. Our results demonstrate, for the first time, highly localized epigenetic changes in the rat brain following acute and chronic morphine exposure. Further work is required to elucidate the potential role of these changes in the formation of tolerance and dependence.

#### Introduction

Morphine is one of the most effective analgesic medications. Morphine, along with other opioids, relieves acute and chronic pain by acting at mu opioid receptors found throughout the central nervous system. Alongside its therapeutic effects, morphine use is also associated with adverse effects such as tolerance and dependence.

Morphine tolerance may originate in part through neuronal processes via activation of mu opioid receptors and subsequent neuroexcitation [1]. Preclinical gene expression profiling studies have demonstrated widespread changes in the transcriptome of the nucleus accumbens and striatum following chronic morphine exposure [2,3], including in genes associated with circadian rhythms, neurotransmitter release, and glucocorticoid receptor signaling. There is also increasing evidence for a role of microglia in tolerance. Microglia are macrophages present within the central nervous system that produce pro-inflammatory cytokines, such as interleukin  $1\beta$  (IL- $1\beta$ ), interleukin 6

(IL-6), and tumor necrosis factor alpha (TNF- $\alpha$ ), following their activation by morphine [4]. IL-1 $\beta$ , IL-6, and TNF- $\alpha$  have been shown to reduce morphine analgesia within five minutes of administration [4], and blockade of IL-1 $\beta$  signaling prolongs morphine-induced analgesia [5], thereby potentially implicating these three cytokines in morphine tolerance. Microglial activation is further implicated in the development of tolerance through increased expression of brain derived neurotrophic factor (BDNF) in the ventral tegmental area (VTA) that facilitates the switch to the dopamine-dependent reward system present in addiction [6]. Notably,  $Bdnf^{-/-}$  knockout mice do not develop tolerance to morphine [7]. Other genes implicated in morphine tolerance include the catechol-O-methyltransferase (COMT) gene through its role in dopamine elimination, whose activity therefore regulates morphine response in mice [8], and the glucocorticoid receptor gene NR3C1, which is epigenetically silenced in the HPA axis following chronic, but not acute, morphine exposure [9].

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Exposure to opiates is also known to induce mitochondrial dysfunction in neuronal and glioma cells [10,11], and chronic exposure is associated with reduced mitochondrial DNA copy number in the rat hippocampus [12]. Furthermore, morphine may induce oxidative stress in the brain [13], and this is known to induce the expression of mitochondrial DNA methyltransferase 1 (mtDNMT1), which regulates the mitochondrial epigenome [14].

Morphine exposure has been demonstrated to affect the epigenetic regulation of genes implicated in addiction and tolerance both in human studies and in animal models. Differential methylation of the *BDNF* [15,16] and opioid receptor Mu 1 (*OPRM1*) [17,18] gene promoters have been reported in the blood of opiate-dependent individuals. Epigenetic changes in the brain may be highly localized, as studies utilizing whole brain tissue have often reported no significant change in global DNA methylation levels following morphine exposure [19,20]. Targeted approaches have proven more insightful, identifying changes in regions of the brain associated with addiction, such as the hippocampus, medial prefrontal cortex, and VTA, in a rat model of heroin self-administration and reward devaluation [21] and post-mortem orbitofrontal cortex brain tissue from former heroin users [22].

However, a significant knowledge gap remains regarding the spatial and temporal epigenetic regulation in the brain of genes that are implicated in tolerance and dependence. In this study, we analyzed epigenetic changes in response to acute and chronic morphine induction in 10 regions of the rat brain reported to be implicated in response to opiates and the formation of addiction: the midbrain contains the VTA, which has been consistently implicated in addiction [23,24]; the pons contains the locus coeruleus, which is key in the integration of opioid and stress

signaling and which is served by innervation from the paraventricular nucleus of the hypothalamus [25]; the inferior and superior colliculus regulate response to opiate withdrawal through reduced activation of mu-opioid receptor signalling [26,27]; the cerebral cortex (containing the dorsomedial prefrontal cortex), hippocampus, and cerebellum are implicated in reinforcement of drug-seeking beheaviors [28–30]; the medulla oblongata is crucial in pain modulation and opiate withdrawal behaviors [31]; and the thalamus, whose function is disrupted in opiate dependence [32,33]. We measured global 5-methylcytosine and 5-hydroxymethylcytosine levels and analyzed the DNA methylation of genes previously identified as implicated in morphine tolerance (*Bdnf, Comt, Il1b, Il6, Nr3c1, and Tnf)* and mitochondrially-encoded genes potentially implicated in mitochondrial dysfunction (*Mt-co1, Mt-co2, and Mt-co3*).

#### Results

#### Study overview

An overview of the study is provided in Figure 1. Male rats were assigned to control, acute challenge, and chronic induction groups (all n = 5). After 10 days, brain tissue was dissected and DNA extracted for epigenetic analysis.

# Gene-specific DNA methylation variability by region of the rat brain

The nuclear-encoded genes *Bdnf*, *Comt*, *Il1b*, *Il6*, *Nr3c1*, and *Tnf* displayed differential variability by region of the brain (Figure 2A-F). *Bdnf* and *Tnf* displayed highly conserved methylation levels between the regions, with mean *Bdnf* methylation



#### Figure 1. Overview of experimental approach.

Rats were assigned to control (A), acute morphine challenge (10 mg/kg one hour post injection, B) and chronic morphine induction (10 mg/kg/day bid for 10 days, C) groups. Saline or morphine was administered twice daily until tissue collection at Day 10.



Figure 2. Variation in DNA methylation by region of the rat brain. A-F: DNA methylation of *Bdnf, Comt, II1b, II6, Nrc31*, and *Tnf* in the cerebellum (Ce), cerebral cortex (CE), hippocampus (Hi), hypothalamus (Hy), inferior colliculus (IC), medulla oblongata (MO), midbrain (Mi), pons (Po), superior colliculus (SC), and thalamus (Th). G-H: global DNA methylation estimated by measurement of *L1-Sutr* and *L1-orf* elements in the same 10 regions of the brain.

levels ranging between 3.0 and 4.6% (Figure 2A) and *Tnf* methylation between 54.0 and 62.5% (Figure 2F). The greatest variability was observed in *Il6* methylation, which displayed low levels (<6.5%) in the cerebellum, cerebral cortex, and hippocampus, but markedly higher methylation (>20%) in the hypothalamus, inferior colliculus, superior colliculus, and the thalamus (Figure 2D). Global DNA methylation levels, estimated using LINE-1 as a surrogate marker (*L1-5utr* and *L1-orf*), were highly consistent between brain regions (Figure 2G-H).

# Morphine induction is associated with gene-specific and global DNA methylation changes in the rat brain

Acute morphine exposure (10 mg/kg, one hour post injection) was associated with significantly increased methylation of *Il6* in the pons (11.6 vs. 14.3%, *P* value < 0.05) and *Nr3c1* in the cerebellum (1.0 vs. 1.2%, *P* value = 0.03), and significantly decreased methylation of *Bdnf* in the pons (3.9 vs. 3.3%, *P* value = 0.03) and *Il1b* in the hippocampus (76.5 vs. 71.8%, *P* value = 0.02) (Table 1, Figure 3). Chronic morphine exposure

(10 mg/kg/day bid for 10 days) was associated with significantly increased methylation of *ll1b* in the medulla oblongata (64.8 vs. 68.4%, *P* value = 0.03) and *Nr3c1* in the hippocampus (1.0 vs. 1.6%, *P* value = 0.02), and significantly decreased methylation of *Bdnf* (3.9 vs. 3.1, *P* value = 0.02) and *Comt* in the pons (77.3 vs. 66.9%, *P* value < 0.0001).

Global DNA methylation levels, estimated through measurement of *Line1* methylation, were significantly lower in the superior colliculus following both acute (*L1-orf*, 82.5 vs. 81.9%, *P* value < 0.05) and chronic morphine exposures (*L1-orf*, 82.5 vs. 81.3%, *P* value = 0.003), and higher in the hypothalamus following chronic exposure (*L1-5utr*, 53.7 vs. 54.9%, *P* value = 0.04) (Figure 4).

# Nuclear 5-hydroxymethylcytosine levels are altered following morphine induction

We measured global 5-hydroxymethylcytosine (5hmC) levels in each of the ten regions of the brain following acute and chronic morphine induction. In the control rats, 5hmC levels



Figure 3. Differential gene-specific DNA methylation in response to morphine exposure. Gene-specific changes in DNA methylation by morphine exposure (control, acute, and chronic) are illustrated, with significant differences indicated (*P* value \* < 0.05, \*\* < 0.01, \*\*\*\* < 0.001, \*\*\*\* < 0.001).



Figure 4. Global DNA methylation levels following acute and chronic morphine exposure. Global DNA methylation levels were estimated through measurement of L1-Sutr and L1-orf elements in 10 regions of the rat brain. Regions displaying significant changes are illustrated (P value \* < 0.05, \*\* < 0.01, \*\*\* < 0.001, \*\*\* \* < 0.0001).

varied by brain region (Table 1). The lowest levels of 5hmC were observed in the hippocampus (0.34%) and thalamus (0.37%), with the highest in the midbrain (0.79%) and pons (0.69%). Significant changes in 5 hmC levels were identified following chronic morphine induction, but none following acute exposure. The levels of 5 hmC increased in the cerebral cortex (0.50% vs. 0.78%, P value = 0.002), hippocampus (0.34% vs. 0.43%, P value = 0.01) and hypothalamus (0.40% vs. 0.54%, P value = 0.008), while they decreased in the midbrain (0.79% vs. 0.36%, *P* value < 0.0001) (Figure 5).

### Morphine induction is not associated with alterations in DNA methylation of mitochondrially-encoded genes

We measured DNA methylation of three mitochondriallyencoded genes (Mtco1, Mtco2, and Mtco3) following morphine exposure. Methylation levels of the three genes were uniformly low across the 10 brain regions, with mean levels ranging between 0.8 (Mtco2, cerebral cortex and pons) and 2.7% (*Mtco1*, cerebral cortex) (Table 2). No significant effect of acute or chronic morphine induction was observed on methylation of the three genes in any of the brain regions. The most substantial changes in methylation were observed for Mtco1 in the cerebral cortex following acute (2.7 vs. 1.8%, P value = 0.23) and chronic (2.7 vs. 1.9%, *P* value = 0.29) morphine induction.

### Discussion

In this study, we investigated the effect of acute and chronic morphine exposures upon nuclear and mitochondrial DNA methylation in ten regions of the rat brain known to be affected

Table 1. Nuclear DNA methylation in the rat brain following morphine induction.										
Region	Treatment	Bdnf	Comt	ll1b	116-1	Nr3c1	Tnf	L1-5utr	L1-orf	5-hmc
Cerebellum	Control	3.0 (0.2)	82.5 (0.3)	64.2 (0.7)	6.3 (0.2)	1.0 (0.0)	62.5 (2.2)	53.3 (0.1)	80.4 (0.5)	0.48 (0.07)
	Acute	3.0 (0.2)	83.6 (0.5)	66.2 (1.3)	5.9 (0.3)	1.2 (0.1)	63.2 (2.8)	53.8 (0.3)	82.9 (0.3)	0.51 (0.06)
	Chronic	3.5 (0.4)	82.4 (0.3)	64.8 (0.4)	6.0 (0.2)	1.1 (0.2)	65.3 (0.9)	53.5 (0.1)	82.4 (0.2)	0.63 (0.03)
Cerebral cortex	Control	3.8 (0.2)	75.8 (1.3)	74.1 (0.7)	5.5 (0.2)	5.7 (1.7)	56.4 (1.3)	53.7 (0.4)	79.7 (0.5)	0.50 (0.06)
	Acute	4.1 (0.4)	74.8 (0.9)	76.1 (1.0)	8.2 (1.8)	3.1 (1.4)	55.3 (1.4)	53.3 (0.2)	80.0 (0.7)	0.54 (0.03)
	Chronic	3.8 (0.4)	77.5 (0.5)	74.6 (2.2)	5.5 (0.5)	4.7 (0.9)	52.8 (1.2)	53.6 (0.2)	79.3 (0.7)	0.78 (0.03)
Hippocampus	Control	4.3 (0.4)	72.4 (1.0)	76.5 (1.3)	6.5 (0.4)	1.0 (0.1)	57.1 (1.0)	54.8 (0.6)	82.2 (0.2)	0.34 (0.02)
	Acute	3.5 (0.2)	70.0 (1.1)	71.8 (1.0)	5.7 (0.5)	1.2 (0.1)	57.5 (1.8)	53.7 (0.2)	83.2 (0.2)	0.35 (0.01)
	Chronic	3.4 (0.2)	71.9 (0.2)	74.8 (0.7)	6.4 (0.3)	1.6 (0.2)	58.9 (1.0)	54.0 (0.4)	82.5 (0.2)	0.43 (0.01)
Hypothalamus	Control	3.6 (0.1)	65.1 (0.8)	70.1 (0.9)	21.8 (1.1)	1.1 (0.1)	59.4 (1.5)	53.6 (0.5)	81.1 (0.2)	0.40 (0.02)
	Acute	3.8 (0.2)	65.2 (1.5)	68.9 (2.1)	16.4 (2.7)	1.3 (0.2)	55.9 (1.4)	54.0 (0.6)	81.5 (0.4)	0.46 (0.02)
	Chronic	3.7 (0.3)	65.0 (0.8)	68.0 (1.2)	20.4 (0.9)	1.1 (0.1)	58.9 (1.1)	54.3 (0.7)	83.4 (0.3)	0.54 (0.03)
Inferior colliculus	Control	4.6 (0.3)	73.4 (0.5)	71.1 (3.4)	23.0 (0.5)	1.2 (0.2)	62.0 (2.1)	54.5 (0.5)	82.6 (0.1)	0.57 (0.1)
	Acute	3.9 (0.4)	72.6 (0.9)	72.4 (3.8)	22.5 (1.2)	1.3 (0.1)	62.5 (1.7)	54.2 (0.4)	80.0 (0.5)	0.62 (0.07)
	Chronic	4.0 (0.4)	72.7 (0.5)	76.1 (0.7)	24.0 (0.5)	1.3 (0.1)	63.7 (1.3)	53.6 (0.6)	77.5 (2.2)	0.79 (0.04)
Medulla oblongata	Control	3.7 (0.4)	69.8 (2.3)	64.8 (1.1)	13.7 (1.4)	1.0 (0.1)	54.0 (1.5)	53.4 (0.3)	80.1 (0.5)	0.51 (0.05)
	Acute	3.4 (0.3)	69.1 (1.1)	65.7 (3.6)	13.2 (0.9)	1.1 (0.1)	53.0 (1.5)	54.5 (0.6)	81.8 (0.2)	0.56 (0.07)
	Chronic	3.8 (0.2)	67.3 (0.8)	68.4 (0.9)	14.6 (0.3)	0.9 (0.1)	56.5 (0.4)	53.9 (0.4)	82.7 (0.1)	0.66 (0.08)
Midbrain	Control	3.3 (0.1)	66.8 (0.9)	76.6 (1.7)	18.8 (1.8)	1.8 (0.5)	58.8 (1.7)	55.4 (0.6)	81.9 (0.2)	0.79 (0.05)
	Acute	3.7 (0.4)	68.1 (1.1)	72.0 (1.8)	19.3 (0.9)	1.9 (0.4)	57.2 (1.8)	53.3 (0.4)	81.8 (0.4)	0.72 (0.03)
	Chronic	3.2 (0.3)	68.2 (1.0)	73.6 (2.0)	21.3 (1.2)	1.6 (0.3)	60.6 (2.1)	54.6 (0.7)	80.7 (0.1)	0.36 (0.01)
Pons	Control	3.9 (0.2)	77.3 (1.1)	74.3 (0.7)	11.6 (0.9)	3.9 (1.1)	55.5 (0.6)	53.6 (0.4)	83.1 (0.3)	0.69 (0.09)
	Acute	3.3 (0.1)	71.6 (2.7)	75.0 (1.4)	14.3 (0.7)	3.6 (0.8)	54.3 (1.1)	53.8 (0.3)	82.3 (0.1)	0.58 (0.05)
	Chronic	3.1 (0.2)	66.9 (0.8)	72.5 (1.6)	12.8 (1.4)	3.7 (1.1)	56.6 (2.5)	54.9 (0.3)	78.5 (0.2)	0.57 (0.06)
Superior colliculus	Control	3.7 (0.2)	74.8 (0.4)	60.3 (4.7)	24.6 (1.4)	1.2 (0.2)	57.0 (2.1)	53.9 (0.4)	79.7 (0.3)	0.54 (0.04)
	Acute	3.3 (0.3)	73.4 (1.8)	59.4 (3.6)	21.4 (1.8)	1.8 (0.5)	57.9 (1.6)	53.9 (0.3)	79.0 (0.2)	0.53 (0.03)
	Chronic	3.2 (0.3)	74.1 (0.7)	58.4 (2.2)	25.1 (0.6)	1.2 (0.1)	60.8 (1.1)	53.6 (0.0)	81.9 (0.2)	0.51 (0.03)
Thalamus	Control	3.6 (0.2)	67.7 (0.6)	73.5 (0.8)	21.0 (2.0)	1.7 (0.3)	57.7 (1.1)	54.4 (0.3)	82.8 (0.1)	0.37 (0.01)
	Acute	3.4 (0.1)	67.1 (1.0)	72.9 (2.4)	20.1 (2.2)	1.8 (0.4)	57.9 (2.4)	54.7 (0.1)	81.3 (0.2)	0.36 (<0.01)
	Chronic	3.6 (0.3)	67.9 (0.8)	72.2 (1.0)	22.2 (0.8)	2.0 (0.4)	60.1 (1.3)	53.5 (0.2)	81.7 (0.4)	0.37 (<0.01)

Mean DNA methylation levels of six genes (Bdnf, Comt, II1b, II6, Nr3c1, and Tnf), global DNA methylcytosine (L1-5utr and L1-orf) and global 5-hydroxymethylcytosine (5hmC) are provided for the 10 regions of the rat brain that were analyzed, with standard error of the mean in brackets.

Region	reatment	IVITCO I	IVITCO2	IVITCO3		
Cerebellum	Control	2.1 (0.1)	0.9 (0.1)	1.1 (0.1)		
	Acute	2.0 (0.2)	1.0 (0.1)	1.2 (0.1)		
	Chronic	2.1 (0.1)	0.9 (0.1)	1.1 (0.1)		
Cerebral cortex	Control	2.7 (0.6)	0.8 (0.1)	1.1 (0.1)		
	Acute	1.8 (0.3)	0.9 (0.2)	1.1 (<0.01)		
	Chronic	1.9 (0.4)	0.8 (0.1)	1 (0.1)		
Hippocampus	Control	1.9 (0.1)	1.0 (0.1)	1.2 (0.1)		
	Acute	1.9 (0.2)	0.8 (0.1)	1.3 (0.1)		
	Chronic	1.8 (0.1)	0.8 (0.1)	1.1 (0.1)		
Hypothalamus	Control	2.0 (0.1)	0.9 (0.1)	1.1 (0.1)		
	Acute	2.1 (0.2)	0.9 (0.1)	1.1 (0.1)		
	Chronic	1.9 (0.2)	0.7 (0.1)	1.0 (0.1)		
Inferior colliculus	Control	2.2 (0.1)	1.0 (0.1)	1.3 (0.2)		
	Acute	2.2 (0.1)	0.9 (0.1)	1.2 (0.1)		
	Chronic	2.0 (0.1)	0.8 (0.1)	1.1 (0.1)		
Medulla oblongata	Control	2.4 (0.3)	1.0 (0.1)	1.1 (0.1)		
	Acute	2.0 (0.2)	0.8 (0.1)	1.1 (0.1)		
	Chronic	2.0 (0.2)	0.8 (0.1)	1.1 (0.1)		
Midbrain	Control	1.9 (0.2)	0.9 (0.1)	1.2 (0.2)		
	Acute	2.2 (0.4)	0.9 (0.1)	1.1 (0.1)		
	Chronic	2.0 (0.2)	1.0 (0.1)	1.1 (0.1)		
Pons	Control	1.9 (0.3)	0.8 (0.1)	1.0 (0.2)		
	Acute	2.3 (0.4)	0.8 (0.1)	1.6 (0.5)		
	Chronic	2.6 (1.0)	0.8 (0.1)	1.1 (0.1)		
Superior colliculus	Control	2.1 (0.1)	1.0 (0.1)	1.3 (0.1)		
	Acute	2.4 (0.2)	0.8 (0.1)	1.3 (<0.01)		
	Chronic	2.1 (0.2)	0.9 (0.1)	1.3 (0.1)		
Thalamus	Control	2.1 (0.1)	1.0 (0.1)	1.3 (0.2)		
	Acute	1.9 (0.1)	0.9 (0.1)	1.1 (<0.01)		
	Chronic	1.8 (0.2)	1.0 (0.3)	0.9 (0.1)		

Table 2. DNA methylation of mitochondrial-encoded genes in the rat brain following morphine induction.

by opiates. Brain region-specific changes were identified in global 5-methylcytosine and 5-hydroxymethylcytosine content, and the *Bdnf*, *Comt*, *Il1b*, *Il6*, and *Nr3c1* genes in response to morphine exposure. We did not observe differential

methylation within the mitochondrial genome. To our knowledge, our study is the first to identify region-specific epigenetic changes in the brain in response to morphine exposure.

Morphine induces the dopaminergic mesolimbic pathway in the ventral tegmental area (VTA) within the midbrain. This region of the brain has a high concentration of dopaminergic neurons and is considered to be crucial in addiction. We observed significantly decreased global DNA methylation in the superior colliculus, estimated through analysis of *Line1*, and a highly significant decrease in 5 hmC content in the midbrain. Together, these observations demonstrate substantial epigenetic changes in this important region of the brain with morphine exposure.

The 5hmC modification is approximately 10-fold more abundant in the brain than other tissues [34], increasing during neuronal development but also implicated in age-related neurodegeneration [35]. While 5hmC is an intermediate in active DNA demethylation, 5mC and 5hmC levels are not inherently correlated [36] and therefore our observations of decreased 5mC and 5hmC are not contradictory. Furthermore, our results could also be the product of the direct role of 5hmC in RNA splicing [37] and gene expression through inhibition of chromatin remodelling [38], which may offer an alternative explanation for our observations. TET1 has been demonstrated to be downregulated in response to cocaine administration, leading to locus-specific decreases in 5hmC [39]. Whilst other studies have reported no change in global 5hmC content in response to heroin and cocaine exposure [19,20,39], they have focused upon a single region (nucleus accumbens) or utilized homogenized brain tissue and therefore could not exclude the possibility of localized changes in 5hmC in specific regions of the brain. The limitations of such approaches were addressed in the current study. Indeed, a particular strength of this work is



Figure 5. Global 5-hydroxymethylcytosine levels following acute and chronic morphine exposure. Global 5 hmC levels were measured by ELISA in 10 regions of the rat brain, with significant changes illustrated (*P* value \* < 0.05, \*\* < 0.01, \*\*\*\* < 0.001).

Mean DNA methylation levels of three genes (*Mtco1*, *Mtco2*, and *Mtco3*) are provided, with standard error of the mean in brackets.

the analysis of ten different regions of the brain that has facilitated the identification of region-specific changes in DNA methylation. Our findings demonstrated significantly increased 5hmC levels in the cerebral cortex, hippocampus, and hypothalamus, in contrast to observations elsewhere of increased hippocampal 5hmC levels following the cessation of cocaine [40]. Further work is required to elucidate the functional implications of these observations.

We identified differential methylation of three genes (Bdnf, Comt, and Il6) in the pons following morphine exposure. This region, located inferior to the midbrain, regulates respiration and sleep, which are commonly disturbed with morphine use. Animal studies have demonstrated a morphine-induced reduction in acetylcholine in the pons and medulla oblongata [41], thereby further implicating this region in response to the drug. BDNF expression facilitates opiate-associated neural plasticity [42] and the dopamine-dependent response to opiates [6]. Chronic exposure to opiates is associated with increased BDNF levels in the VTA [23], and it has been demonstrated that this increase begins rapidly after initial usage [43]. Other studies have reported increased expression of BDNF in the VTA following morphine withdrawal [24]. Our results provide further evidence for the role of BDNF in the acute response to morphine exposure and suggest that this may not be limited to the VTA. Catechol-O-methyltransferase (COMT) is implicated in dopamine elimination and, subsequently, COMT activity reduces response to morphine [8], while genetic variants in the COMT gene are associated with differential response to morphine as an analgesic [44]. Our observation of decreased Comt methylation with chronic exposure may be in response to increased dopamine signaling and a role in its catabolism. The changes seen in Il6 in the pons may be in accordance with the modulating role that opioids have in inflammatory pathways [45]. Our observation of increased Il6 promoter methylation is in contrast to reports of increased secretion in acute response to morphine in the spinal cord [4] and plasma [46,47]. Notably, however, we also identified regional-specificity in Il6 methylation levels and, therefore, the difference in measured methylation levels may represent alterations in cell composition, such as increased microglial infiltration. As IL-1 $\beta$  and TNF- $\alpha$  are also secreted by activated macrophages in response to morphine [48], but were not differentially methylated in the pons, it is unclear how IL-6 alone may be implicated in response to morphine.

Morphine administration can induce the generation of reactive oxygen species (ROS) by increasing the metabolism of substrates such as dopamine and xanthine oxidase [49]. Elevated ROS levels are known to induce site-specific alterations in DNA methylation by mechanisms such as modulation of the expression of DNA methyltransferases (DNMTs) [50]. While the observed gene-specific changes in DNA methylation are likely to be directly induced by morphine administration, global changes in 5mC and 5hmC content could in part be non-specific effects through increased ROS production.

The study of the mitochondrial epigenome is an emerging field of interest. Alterations in mitochondrial DNA methylation have been demonstrated in cardiovascular disease [51] and in response to environmental exposures [52,53]. It has been speculated that such epigenetic changes may give rise to the altered mitochondrial function observed in drug addiction [14]. However, our study did not identify differential methylation of mitochondrial genes in response to morphine induction. We cannot exclude the possibility that other regions of the mitochondrial epigenome may be differentially methylated in response to exposure, and therefore more comprehensive analysis is required.

Our study contains certain limitations that restrict the inferences that can be made from our observations. In particular, the absence of rat behavioral studies and the lack of RNA and protein samples to analyze gene expression have precluded the study of the functional impact of the observed epigenetic changes. The number of cells obtained from some of the dissected tissue specimens was only sufficient to extract DNA from and, therefore, no analysis of gene expression was possible. Gene-specific analysis of 5 hmC content would also have proven highly insightful, but it is currently prohibitively expensive. Nonetheless, our study offers several important strengths. Firstly, the study of ten different regions of the brain has enabled us to identify region-specific epigenetic changes in response to drug exposure. Furthermore, we have performed a range of epigenetic analyses (nuclear and mitochondrial genespecific DNA methylation and global 5mC and 5hmC content) that have facilitated a broader study of the epigenome in response to morphine exposure. Finally, our study design has enabled the delineation of epigenetic changes associated with acute and chronic morphine exposure.

In conclusion, we have identified gene-specific and global changes in DNA methylation in response to acute and chronic morphine exposure, which were highly localized to specific regions of the brain. Of especial interest, we identified significant changes in 5 hmC content in four of the ten regions, suggestive of wider epigenetic remodeling in response to morphine exposure. We hope that our findings will help inform further studies in the treatment of dependence, including elucidation of the functional consequences of the observed epigenetic changes and how they relate to long-term response to opiates. Additionally, our findings may help facilitate the production of biomarkers of drug tolerance. Given the increase in deaths from opiate overdose within the past decade [54,55], there is urgent clinical need for tolerance biomarkers that could help reduce thousands of avoidable deaths.

#### **Materials and methods**

#### Animals and housing conditions

Male Wistar rats (180–200 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Following shipment, rats were housed in a temperature (22–24 °C) and humidity (60%) controlled environment on a 12-hour light/dark cycle (lights on at 7:00 AM) for three days to allow for acclimatization prior to experimental studies. The animals were given free access to food and water *ad libitum*. All efforts were made to reduce the number of animals used and minimize their suffering. Experimental study groups were assigned randomly. All procedures were approved by the Laboratory Animal Use Committee of Shanghai Jiao Tong University School of Pharmacy.

#### Drugs

Morphine hydrochloride was purchased from Shenyang First Pharmaceutical Co., Ltd. (Shenyang, Liaoning, China) and Maybridge Chemicals (Cornwall, UK). CBIO was freshly dissolved in sterile normal saline solution (Sinopharm Group Chemical Reagent Co., Ltd.) with the pH adjusted to 7.3–7.5 with 1 M NaOH solution.

### **Morphine induction**

Male rats were assigned into three groups (each n = 5) receiving alternative treatment regimens: saline/saline (Group A, control); saline/morphine (Group B, acute challenge, 10 mg/kg); and morphine/morphine (Group C, chronic induction). For morphine chronic induction, the first treatment period consisted of subcutaneous injection of saline (Groups A and B, 10 ml/kg) or morphine (Group C, 10 mg/kg) twice daily at 12-hour intervals for nine

Table 3. PCR and pyrosequencing primer sequences.

consecutive days [56]. On Day 10, the second treatment was performed by a single subcutaneous injection of saline (Group A) or morphine (Groups B and C, 10 mg/kg) (Figure 1). Previous work has demonstrated tolerance to morphine is developed within 7–10 days, with epigenetic changes observable in the rodent brain 5–7 days following first treatment [57,58].

#### Sample preparation and DNA extraction

The rats were decapitated on Day 10, one hour after the last administration. Brains were removed from the skull and ten brain regions (cerebellum, cerebral cortex, hippocampus, hypothalamus, inferior colliculus, midbrain, pons, medulla oblongata, superior colliculus, and thalamus) were rapidly dissected, frozen on dry ice, and kept at  $-80^{\circ}$ C until processed. DNA was extracted from the tissues using the Wizard Genomic DNA purification kit (Promega, Madison, WI) according to the

Bdmf       Promoter IV       Forward primer (5' to 3) Reverse biolin primer (5' to 3) Sequencing primer (5' to 3) Sequencing primer (5' to 3)       TITITAMITIAANIGAGI CACAADAANITIATIATIANATAMA GTITITAGATAGI GTIGGCGGATATATITIC/GTAGI GTIGGCGGATATATITIC/GTAGI GTIGGCGGATATATITIC/GTAGI GTIGGCGGATATATITIC/GTAGI GTIGGCGGATATATITIC/GTAGI GTIGGCGGATATAGGI TAGGGGTGGACGGGGGCGGGG CTGGCGGATATAGGI GTIGGTGGGGGGGGGGGGGGGGGGGGGGG	Gene name	Gene region	Primer	Sequence
Cont       Reverse biotin primer (5' to 3)       CAACAAAAAAAAAATAATAATAATAATAAAA         Sequence analyzed       C/TGTCAGATATATTATATAATAATAAA         Comt       Promoter P1       Reverse biotin primer (5' to 3)       CTATTCCCCCCATACT         Ni3c1       GR11n       Reverse biotin primer (5' to 3)       CTATGCTCCCCCATACCT         Ni3c1       GR1n       Reverse biotin primer (5' to 3)       CTAAAAACTCCCCCCCTCCCCCCTATACCT         Ni3c1       GR1n       Reverse biotin primer (5' to 3)       CTAAAAACTCCCCCCCCTCCCCCCCCCCCCCCCCCCCC	Bdnf	Promoter IV	Forward primer (5' to 3')	TTTTTAGTTTTTGTTTAGATTAAATGGAGT
Sequencing primer (5' to 3)       GTITTATIGAAGG         Cont       Promoter P1       Forward primer (5' to 3)       GTITTATIGAAGG         Kisker       Forward primer (5' to 3)       GTGTGCTGAGTTATITTC/GTTATIGG         Nisker       GR110       Sequencing primer (5' to 3)       GTGGTTAGTGGGGGTGTGGGGGGGGCGGGGGGGGGGGGG			Reverse biotin primer (5' to 3')	CAACAAAAAAATTAAATTATTAATAATAAA
Sequence analyzedCfGTGC/TGAGTTTTTTGCGTGAGTContPromoter PIForward primer (5' to 3')GTTGGTGAGTGAGTGGGGGGGGGGGGGGGGGGGGGGGG			Sequencing primer (5' to 3')	GTTTTTATTGAAGG
Cont   Promoter P1   Forward primer (5' to 3)   CTTCTGTGATTTGGAGTTAGGT     Nr3c1   GR110   Reverse biotin primer (5' to 3)   CTGGTTGGGGGTGTAGGGGGGGTGGGGGGGGGGGGGGGG			Sequence analyzed	C/TGTGC/TGAGTATTATTTTC/TGTTATG
Reverse biotin primer (5' to 3)   CTCATCCTCCCCCATTACCT     Nr3c1   GR1 <sub>10</sub> Sequence analyzed   CTGGTTGTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	Comt	Promoter P1	Forward primer (5' to 3')	GTTTGTTGTAGTTTGGAGTTAGGT
Sequencing primer (5' to 3)TATGGTTTAGTGTGGNr3c1GR110Forward primer (5' to 3)CTGGTTGGGGGTAGGGGGGGGGGGGGGGGGGGGGGGGGG			Reverse biotin primer (5' to 3')	CTCATCCTCCCCATTACCT
Sequence analyzed   CTGGTTGTGGGTGTAGGGGGGGTGAGGGGGGGT     Nr3c1   GR110   Forward primer (5' to 3)   CTAAAAACTCTCCCCCCCC     IIIb   Promoter   Forward primer (5' to 3)   CTAAAAACTCTCCCCCTCCCC     IIIb   Promoter   Forward primer (5' to 3)   CTACTGTTGTGGTTCTGTCTGCGCGGGGGGG     IIIb   Promoter   Forward primer (5' to 3)   ATAAAATCATTACCCATAGTTGCTGTCTGCC     IIIb   Promoter   Forward primer (5' to 3)   ATAAAATCATTACCCATAGAAAAAA     Sequencing primer (5' to 3)   ATAAAATCATTACCCATAGTTTGGTGTGTCTGC     IIIb   Promoter   Forward primer (5' to 3)   ATAAAATCATTACCCATAGTTTGGTGTGTGAGGGAGGA     IIIb   Promoter   Forward primer (5' to 3)   CTAGCTGTGTGGTGGCGCTGTGTAGGGGGGGG     IIIb   Promoter   Forward primer (5' to 3)   CTAGCTGTGTGGTGGCGCTGTAAGGAAAAAAA     IIIb   Promoter   Forward primer (5' to 3)   CTAGCTGTGGTGGTGGTGTAAGGA     IIIb   Promoter   Forward primer (5' to 3)   CTAGCTGTGGTGGTGGTGTGTAGGGGGGGG     IIIb   Promoter   Forward primer (5' to 3)   CTAGCTGTGGTGGTGGTGTAAGGGA     IIIb   Promoter   Forward primer (5' to 3)   CTAGCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT			Sequencing primer (5' to 3')	TATGGTTTAGTGTGG
M3C1   GR110   Forward primer (5' to 3)   GGTTGGTAAAAGTTTGTTAAGTT     II/b   Promoter   Sequencing primer (5' to 3)   GTTGTAAAGTTTGTTAAGTT     II/b   Promoter   Forward primer (5' to 3)   GTTGTAAAGGAAGTTTGTC/TGC/CTGC/CGC/CC/CGC     II/b   Promoter   Forward primer (5' to 3)   GTTATAAGGAAGTTTGTATGGAGAG     II/b   Promoter   Forward primer (5' to 3)   ATGATAAGGAAGTTTGTTAGTGGAGAG     II/b   Promoter   Forward primer (5' to 3)   ATGATATGACTGTGC/CACTAGTTTGTAATGA     II/b-1   Promoter   Forward primer (5' to 3)   CAAACATCCCCAATGTCGATGAGAG     II/b-2   Promoter   Forward primer (5' to 3)   CAAACATCCCCAATGTCAATGAG     II/b-2   Promoter   Forward primer (5' to 3)   CAAACATCCCCAATGTCATTTAT     Sequence analyzed   C/TGGTTATATGGAGAGAGT   Sequence analyzed     II/b-2   Promoter   Forward primer (5' to 3)   CAAACATCCCCAATGTCATTTAT     Sequence analyzed   C/TGGTTATAAGGAGGAGATGA   Sequence analyzed   C/TGGTTTATAGAAAAACGTTATGTGAGAGG     II/b-2   Promoter   Forward primer (5' to 3)   CAAACATCCCCAATGCAAGGG     Sequence analyzed   C/TGGTTTATGGAGAGAAATTGA   Sequence analyzed   C/TGGTATTGGAGAGAGAATTGA     II/b-2   Forward primer (5' to 3)   CAAACATTCCCTAATAAAAAAAAACCTCCCAATGCA   Sequence analyzed   C/			Sequence analyzed	C/TGGTTGTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
Micro   Reverse biotin primer (5' to 3')   CTAAAAACTCTCCCCCCCCCCCCCCCCCCCCCCCCCCC	Nr3c1	GR110	Forward primer (5' to 3')	GGTTGGTAAAAGTTTGTTAAGTT
Sequencing primer (5' to 3)   GTTATTTACTATT     IIIb   Promoter   Forward primer (5' to 3)   GTTATTTACTATT     IIIb   Promoter   Forward primer (5' to 3)   ATAAAATCAATTAACCCAAAAAAA     Sequencing primer (5' to 3)   ATAAAATCAATTAACCCAATAAAAA     Sequencing primer (5' to 3)   ATAAAATCAATTAACCCAATAAAAA     III6-1   Promoter   Forward primer (5' to 3)   ATAAATTAATTTTGAATTATT     III6-2   Promoter   Forward primer (5' to 3)   CTAGCTGTTCACTGTTAAATGA     III6-2   Promoter   Forward primer (5' to 3)   CTAGACTTCCAATTTTGATTAATGA     III6-2   Promoter   Forward primer (5' to 3)   CTAGACTTCCCAATCTCATATTTAT     Sequencing primer (5' to 3)   CTAGATTTTTGGTGTAAATGA   Reverse biotin primer (5' to 3)   CTAGATTTTTGGTGTAAATGA     III6-2   Promoter   Forward primer (5' to 3)   CTAGATTTTTGGTGTAATGA     III6-2   Promoter   Forward primer (5' to 3)   CTAGATTTTAAATGAAGAGAT     Sequencing primer (5' to 3)   CTAGATTTTAATGAAGAGAGAT   Sequencing primer (5' to 3)   CTAGATTTTAATTACCCAATATTAATCCAAGAGAGAT     MT-co1   Exon   Forward primer (5' to 3)   ACTTCGTATATAAAAAAAACCATAATTCC     MT-co2   Exon   Forward primer (5' to 3)   ACTTCGTATTAAATAAAAAAAATAATCATCATATAATTAAATTAAATTAAATTAAATTAAATTAAATTAAATTAAATTAAATTAAATTAAATTAAATTAAATTAATTAATTAATTAA     MT-co3		210	Reverse biotin primer (5' to 3')	СТАААААСТСТССССТСССС
Sequence analyzed       C/TGC/TGC/TGT/C/GTTC/TGTC/GTC/TGC/GT         IIIb       Promoter       Forward primer (5' to 3')       ATAAATCAATTAACCCAAAAAAA         Sequence analyzed       C/TGC/TGC/TGTC/C/GTTC/GTC/GT       GATAAAGCAATTAACCCAAAAAAAA         III-0       Promoter       Forward primer (5' to 3')       ATAAATCAATTAACCCAAAAAAAA         III-0       Promoter       Forward primer (5' to 3')       ATGTTTTGAATTATT         III-0       Promoter       Forward primer (5' to 3')       TTGTGATTTTTGGATGTTAAATGA         III-2       Promoter       Forward primer (5' to 3')       TTGTGATTTTTGGATGTTAAATGA         III-2       Promoter       Forward primer (5' to 3')       TTGTGATTTTTGGATGTTAAATGA         III-2       Promoter       Forward primer (5' to 3')       TGTAAAGTCCCCAAGGAT         III-2       Promoter       Forward primer (5' to 3')       TGTAAAGTCCCCAAGGAT         III-2       Promoter       Forward primer (5' to 3')       CAAACATCCCCAAATTTAA         III-2       Promoter       Forward primer (5' to 3')       CAAACATCCCCAATTTAA         III-2       Promoter       Forward primer (5' to 3')       CATGTCTTAAAAAAAAAAACCATCA         III-2       Sequencing primer (5' to 3')       TAAACTCCCTAAATTAAATTGAGGGAGGATTTAA			Sequencing primer (5' to 3')	GTTTATTTTAGTATT
IIIb   Promoter   Forward primer (S' to 3)   TGTATAAGGAACTITGATTGGAGAG     IIIb   Promoter   Forward primer (S' to 3)   ATGAAATTAACCAATTAACCCAAAAAAA     IIIc-1   Promoter   Forward primer (S' to 3)   ATGATTIGATTATT     IIIc-1   Promoter   Forward primer (S' to 3)   C/TGGGTTIGCTGCCCCATAGTTITCTTCCTCCCT/TGTTTA     IIIc-1   Promoter   Forward primer (S' to 3)   CAAACATCCCCAATTAATGA     IIIc-2   Promoter   Forward primer (S' to 3)   CAAACATCCCCAATTAATTAT     Sequence analyzed   C/TGTATAT   CAAACATCCCCCAATTAATTAT     IIIc-2   Promoter   Forward primer (S' to 3)   CAAACATCCCCAATTAATGA     IIIc-2   Promoter   Forward primer (S' to 3)   CAAACATCCCCAATTTAATTAT     Sequence analyzed   C/TGTATAT   CAAACATTCCCAATTTAATTAT     Sequence analyzed   C/TGTATTTA   CAAACATTTCAATTAATTAT     Sequence analyzed   C/TGTATTTAT   CAACATTTCAATTTAT     Traf   Promoter   Forward primer (S' to 3)   CGAATTTGATAGAGAGG     Traf   Promoter   Forward primer (S' to 3)   CATCCTTATATTTAT     Sequencing primer (S' to 3)   CATCCTATATAAAAAAAAAACTACCTAATAATACGATAGATGA     MT-co1   Exon   Forward primer (S' to 3)   AATTTTGATAGAAAAAAAAACCCCCCAATAAAAAAAAAA			Sequence analyzed	
Instruction     Formodel     Reverse biotin primer (5' to 3')     ATAAAATCAATTAACCCCAAAAAAAA       Ide-1     Promoter     Forward primer (5' to 3')     ATGTTTTGGATTATT       Ide-1     Promoter     Forward primer (5' to 3')     TGTGGATTTGCGTCCCTCAGTTTTCCTCCCCC/TGTTTA       Ide-1     Promoter     Forward primer (5' to 3')     TGTGGATTTGGATGTAAATGA       Ide-2     Promoter     Forward primer (5' to 3')     TGTGATTTTTGGATGTAAATGA       Ide-2     Promoter     Forward primer (5' to 3')     TGTGATTTTTGGATGTAAATGA       Ide-2     Promoter     Forward primer (5' to 3')     TGTGATTTTTGGATGTAAATGA       Ide-2     Promoter     Forward primer (5' to 3')     TGTGATTTTTGGTGAGG       Ide-2     Promoter     Forward primer (5' to 3')     CAAACATCCCCAATCCCATATTAT       Sequence analyzed     C/TGATTAT     CAAACATCCCCAATCCCATATTAT       Sequencing primer (5' to 3')     CAAACATCCCCAATCCCATATTAT       Sequence analyzed     C/TGATTTTAA     CAAACATCCCCAATCCCATATTTCGTGAGG       MT-co1     Exon     Forward primer (5' to 3')     ACTCCTAATAATAAAAAAACCATAATCCC       Sequence analyzed     C/TGGATTGGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGAATTTTTT	ll1b	Promoter	Forward primer (5' to 3')	TGTATAAGGAAGTTTGATTGGAGAG
III6-1   Promoter   Forward primer (5' to 3')   ATGTITIGAATTATT     II6-1   Promoter   Forward primer (5' to 3')   CATAGGGTTIGCTGTCCACTAGTTTTTAT     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCGTATATGA     II6-2   Promoter   Forward primer (5' to 3')   TIGGTTTTTGGATGTTAAATGA     II6-2   Promoter   Forward primer (5' to 3')   TIGGTATTTTGGATGTAAATGA     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCCATATTTAT     Sequencing primer (5' to 3')   CAAACATCCCCCAATCCATATTTAT   Everse biotin primer (5' to 3')     Thf   Promoter   Forward primer (5' to 3')   CAGACATCCCCAATCCATATTTAT     Sequencing primer (5' to 3')   CAGACATCCCCCAATCCCATATTTAT   Everse biotin primer (5' to 3')     MT-co1   Exon   Forward primer (5' to 3')   ACTICGTAATAAAAAAACCATAATCC     Sequencing primer (5' to 3')   ACTICGTAATAAAAAAAACCCCCC   Sequencing primer (5' to 3')   AATGGAATTGGAATGGAATGAATTAAT     MT-co2   Exon   Forward primer (5' to 3')   TAATGGAATTAGTTAAATAAAAAAAACCCCCC     Sequencing primer (5' to 3')   ATAGTTTAAATTAATTAGGGGAATTGAATTAATA     Sequencing primer (5' to 3')   TAATGGAATGGAATGAATTTAATA     MT-co2   Exon   Forward primer (5' to 3')   TAATGGATTTAAATTAATTAATTAATTAAATTAAAAAAAA	110	Tomoter	Reverse biotin primer (5' to 3')	ΑΤΑΔΔΑΤΓΑΔΓΓΓΑΔΑΔΔΔΔΔΔ
Iber analyzed   C/IGGGGTTIGCTGTCCCTC/TGTTTA     Iber analyzed   C/IGGGGTTGCTGTCCCATAGTTTCTCTCCCCTC/TGTTTA     Iber analyzed   C/IGGGGTTGTGTGTCCCCATGTTTA     Iber analyzed   C/IGGGGTTGTGTGTCCCCATGTTTA     Iber analyzed   C/IGTGGTTGTGTGTCCCCATGTTA     Iber analyzed   C/IGTGGTTGTAATGA     Iber analyzed   C/IGTGGTTGTAATGA     Iber analyzed   C/IGTGATTTTTGGATGTTAAATGA     Iber analyzed   C/IGTGATGTTAAATGA     Iber analyzed   C/IGTGATGTTAAATGA     Iber analyzed   C/IGTGATGTTAAATGA     Iber analyzed   C/IGTATAT     Iber analyzed   C/IGTATGTTAAATGA     Iber analyzed   C/IGTATAT     Iber analyzed   C/IGTATGGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG			Sequencing primer (5' to 3')	ΔΤGTTTTGΔΔΤΤΔΤΤ
II6-1   Promoter   Forward primer (5' to 3')   TIGGATITITIGATGITAAATGA     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     II6-2   Promoter   Forward primer (5' to 3')   TIGTGATTTTTGGATGTTAAATGA     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     Sequencing primer (5' to 3')   CAAACATCCCCAATCTCATATTAT   Sequencing primer (5' to 3')   CAAACATCCCCAATTGGAGG     II6-2   Promoter   Forward primer (5' to 3')   GGATGTAAAAAAAAAACCATAATCC   Sequencing primer (5' to 3')   ACTCCTCATATAAAAAAAAACCATAATCC     Sequencing primer (5' to 3')   ACTCCTAATAAAAAAAAACACCCCC   Sequencing primer (5' to 3')   AAATCTCCAAAAAAAAAACACCCCC     MT-co2   Exon   Forward primer (5' to 3')   AAATCTTTAAATTAGGTTAAAATACCAAAAATATCAAAAAAATACCAAAAAAAA			Sequence analyzed	
Indext   Promoter   Promoter   Promoter   Promoter   CAAACATCCCCAATCTCATATTAT     II6-2   Promoter   Forward primer (5' to 3')   TIGGATTTTTGGATGTTAAATGA     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     Sequence analyzed   C/TGATTTTA   Sequencing primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     Sequence analyzed   C/TGATTTTA   Sequencing primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     Trif   Promoter   Forward primer (5' to 3')   ACTTCCTTAATAAAAAAAAAAAAAAAAAAAAAAAAAAA	116 1	Bromotor	Ecriment (5' to 2')	
II6-2   Promoter   Sequencing primer (5' to 3') Sequencing primer (5' to 3')   CHARCHTCCCCANTCHTANATGA (//GTTATAT     II6-2   Promoter   Forward primer (5' to 3') Sequence analyzed   C//GTTATAT     II6-2   Promoter   Forward primer (5' to 3') Sequencing primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCTCATTTAT     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCCAATCTCATTTAT     II6-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCCAATCTCATTTAT     II6-2   Promoter   Forward primer (5' to 3')   GGATTGTTATAGAAATTTGGTGAGG     II6-2   Promoter   Forward primer (5' to 3')   GGATTGTTATAGAAAAAACATTACTC     II6-2   Exon   Forward primer (5' to 3')   AGTTGGAATGGAGAATTA     II6-2   Exon   Forward primer (5' to 3')   TAATCTCTAAAAAAAACAACCCC     Sequencing primer (5' to 3')   AAATTTATTTAGTGGGGATATTCATGTAGATTTAA     II6-2   Exon   Forward primer (5' to 3')   TAATCCTAAAAAAAATAACCATCATAATA     MT-co2   Exon   Forward primer (5' to 3')   TAATCCTAAAAAAAATAACCATCATAATA     Sequencing primer (5' to 3')   GTATATAAAAAAAATTATCATAAATA   Sequencing primer (5' to 3')     MT-co3   Exon   Forward primer (5' to 3')   GTATATAAAAAAAATCATCAAAAAATCC <t< td=""><td>110-1</td><td>FIGHIOLEI</td><td>Polyarea biotin primer (5' to 2')</td><td></td></t<>	110-1	FIGHIOLEI	Polyarea biotin primer (5' to 2')	
Bequeticing primer (5' to 3')Transform models in AARIGA1/6-2PromoterForward primer (5' to 3')TIGATITITITGGATGTTAAATGA1/6-2PromoterForward primer (5' to 3')CAAACATCCCCAATCTCATATTAT1/6-2PromoterForward primer (5' to 3')CAAACATCCCCAATCTCATATTAT1/6-2PromoterForward primer (5' to 3')CAAACATCCCCAATCTCATATTAT1/6-2PromoterForward primer (5' to 3')CAGATGTCAAAAAAAAAACCATAATCTC1/6-2PromoterForward primer (5' to 3')ACTTCCTTAATAAAAAAAAAACCATAATCTC1/6-2Sequencing primer (5' to 3')ACTTCCTTAATAAAAAAAAAACACCCC1/6-20ExonForward primer (5' to 3')ACTTGGAATAGAATGGA1/6-20ExonForward primer (5' to 3')TAACTCTAAAAAAAAAACACCCCC1/6-20ExonForward primer (5' to 3')TAATCCTAAAAAAAAAACACCCCC1/6-20ExonForward primer (5' to 3')TAATCGTTAAAAAAAAAACTAACTCATAAATAA1/6-20ExonForward primer (5' to 3')TAATCGTTAAAAAAAATAACTCATAAATA1/6-20ExonForward primer (5' to 3')TAATCATTTAATTTTATTTTTTGTGGGACATTTTAA1/6-20ExonForward primer (5' to 3')TAATCATTAATTTTTTTTTTTTTTTTTTTTTTTTTTTT			Sequencing primer (5 to 5)	
Ib-2   Promoter   Forward primer (5' to 3')   TIGTARTITTGGATGTTAAATGA     Ib-2   Promoter   Forward primer (5' to 3')   CAAACATCCCCAATCTCATATTAT     Sequence analyzed   C/TGATTTTA     Inf   Promoter   Forward primer (5' to 3')   GGATTGTTATAGAATTGAGAGG     Inf   Promoter   Forward primer (5' to 3')   GGATTGTTATAGAATTTGGTGAGG     MT-co1   Exon   Forward primer (5' to 3')   ACTTCCTTAATAAAAAAAACCATAATCC     Sequence analyzed   C/TGTATTTGGAGAGAGAT     MT-co1   Exon   Forward primer (5' to 3')   AGTTGGATGGAATGGAATAGA     MT-co2   Exon   Forward primer (5' to 3')   TAACTCCTAATAAAAAAAACCCCCC     Sequence analyzed   C/TGGAATTGGAGGGAATATTC/TGTAGAGTTTAA     MT-co2   Exon   Forward primer (5' to 3')   TAATCTCTAAAAAAAAAACCCCCC     Sequence analyzed   C/TGGAATTGGAGGGAATATTC/TGTAGAGTTTAA     MT-co3   Exon   Forward primer (5' to 3')   TAATGATTTAATTGTGGAGGAATTT     MT-co3   Exon   Forward primer (5' to 3')   TAATCCTAATAAAAAAAACCCATAATAA     Sequence analyzed   C/TGGAATTAGATTAAATAACTCATAAATAA     Sequence analyzed   C/TGGAATTAGATAATAACTCATAAATAA     MT-co3   Exon   Forward primer (5' to 3')   AATATATAAAAAAAATACTCATAAAAAAATACCAATATA     Sequence analyzed   C/TGGAATTAGATTTATTTTATTT			Sequencing primer (5 to 5)	
Id=2   Promoter   Promoter   Promoter   Promoter   Promoter     Tinf   Promoter   Forward primer (5' to 3')   CAACATCCCAATTTATTTAT     Tinf   Promoter   Forward primer (5' to 3')   GATTGTTATAGAATTTTGGTGAGG     MT-co1   Exon   Forward primer (5' to 3')   ACTTCCTTAATAAAAAAAACCCTAATTCC     MT-co2   Exon   Forward primer (5' to 3')   ACTTCCTAAAAAAAAAACCCCC     MT-co2   Exon   Forward primer (5' to 3')   ACTTCCTAAAAAAAAAACCCCC     MT-co3   Exon   Forward primer (5' to 3')   TAACTCCTAAAAAAAAAACCCCC     MT-co3   Exon   Forward primer (5' to 3')   TAACTCCTAAAAAAAAAACCCCC     MT-co3   Exon   Forward primer (5' to 3')   TAACTCCTAAAATAAAAAAAACCCCC     MT-co3   Exon   Forward primer (5' to 3')   TAACCCTAAAATAAAAAAAAACCCCC     MT-co3   Exon   Forward primer (5' to 3')   TAACCCTAAAATAAAAAAAAACCCTAAATA     Sequencing primer (5' to 3')   ATAGAATTTAAATAAAAAAAACCCCC   Sequencing primer (5' to 3')   ATAGAATTTAAATAAAAAAAACCCCC     MT-co3   Exon   Forward primer (5' to 3')   TAACCCTAAAAAAAAAAAAAAACCCTAAAATA   Sequencing primer (5' to 3')   ATAGAATTTTAAAAAAAAACCCCC     L1-utr   -   Forward primer (5' to 3')   GTGTATAAGAATATTAAAAAAAAATACTCAAAAAAAAAA	116.2	Dromotor	Sequence analyzed	
Inference   Sequencing primer (5' to 3')   CAAACITCCCATATTAAT     Traf   Promoter   Forward primer (5' to 3')   GGATTGTTATAGAATTTGGTGAGG     MT-co1   Exon   Forward primer (5' to 3')   ACTTCCTTAATAAAAAAACCATAATTA     MT-co1   Exon   Forward primer (5' to 3')   ACTTCCTTAATGAAAAAAACCCCC     Sequencing primer (5' to 3')   TAAATTTTGGTGGAGATGAATA     MT-co1   Exon   Forward primer (5' to 3')   ACTTCCTTAATGAAAAAAACCCCC     Sequencing primer (5' to 3')   TAATGATTAGGAATAGGATGAATA     Reverse biotin primer (5' to 3')   TAATGCTTAAGAATAGAAAAACCCCC     Sequencing primer (5' to 3')   ACTTCCTTAAAATAAAAAAACCCCC     Sequencing primer (5' to 3')   TAATGATTTAAGTTAATTAGTTGGGG/ATATTC/TGTAGATTTAA     MT-co2   Exon   Forward primer (5' to 3')   TAATGAATTTAAGTTAATAAAAAAAAAATAACTCATAAAATA     Sequencing primer (5' to 3')   TAATGAATTTAATTTATTC/TGAAGAC/TGTTTG     MT-co3   Exon   Forward primer (5' to 3')   ATAGAATTTAAAAAAAAATAACTCAAAAAGGTTT     MT-co3   Exon   Forward primer (5' to 3')   GTATATAAAAAAAGGTTT     MT-co3   Exon   Forward primer (5' to 3')   GTATATAAAAAAAGGTTT     Sequencing primer (5' to 3')   GGTATATATATTTAAAAGGTTAAAAGGTTT   Sequencing primer (5' to 3')     L1-utr   -   Forward primer (5' to 3')   GTATATAAAAAGGTTTT	110-2	Promoter	Forward primer (5 to 3)	
MT-co1   Exon   Forward primer (5' to 3')   GGATTGTTATAGAATTGAAAAAAAACCCC     MT-co1   Exon   Forward primer (5' to 3')   ACTTCCTTAATAAAAAAAACCCC     Sequence analyzed   C/TGGATTGGAGGAGAGAATTGA     MT-co1   Exon   Forward primer (5' to 3')   ACTTCCTAATAAAAAAAACCCCC     Sequence analyzed   C/TGGATTGGAGAGAGAATTGA     MT-co2   Exon   Forward primer (5' to 3')   TAACTTCCTAAAAAAAAACCCCC     Sequencing primer (5' to 3')   TAACTCCTAAAAAAAAAACCCCC     Sequencing primer (5' to 3')   TAACTCCTAAAAAAAAAACCCCC     Sequencing primer (5' to 3')   TAACTCCTAAAAAAAAACCCCC     Sequencing primer (5' to 3')   TAACTCATAAAAAAAAACCCCC     Sequencing primer (5' to 3')   TAACTCATAAAAAAATAACTCATAAATA     MT-co2   Exon   Forward primer (5' to 3')   TAACCATAAAAAAATAACTCAAAAAATAA     MT-co3   Exon   Forward primer (5' to 3')   TAATGATTTAAAAAAAATAACTCAAAAAATTA     MT-co3   Exon   Forward primer (5' to 3')   GTATTATATTTTTATGTTTG     MT-co3   Exon   Forward primer (5' to 3')   GTATTATAAAAAATCCAAAAAGGTT     MT-co3   Exon   Forward primer (5' to 3')   GTATTATATATTTTTATGTTTTTTTTTTTTTTTTTTTT			Reverse blotin primer (5° to 3°)	
Image: Sequence analyzed   C/TGATTTTAA     Tinf   Promoter   Forward primer (5' to 3')   GGATTGTTATAGAATTTGGTGAGG     Reverse biotin primer (5' to 3')   ACTTCCTTAATAAAAAAAACCATAATCTC     Sequencing primer (5' to 3')   TTAAAATTTTGGTGAGGAGAAAAATGA     MT-co1   Exon   Forward primer (5' to 3')   AGTTGGAATAGGATGAATAG     MT-co2   Exon   Forward primer (5' to 3')   TAACTCCTAATAAAAAAACACCCCC     Sequence analyzed   C/TGGAATTGGAATTGGGG/ATATC/TGTAGATTTAA     MT-co2   Exon   Forward primer (5' to 3')   TAATCTTAAAAATTAAAAAACCCCCC     Sequence analyzed   C/TGGAATTTAGTTGGGG/ATATC/TGTAGATTTAA     MT-co2   Exon   Forward primer (5' to 3')   TAACCCTAATAAAAAAATAACTCATAAATA     Sequencing primer (5' to 3')   TAATGATTTAAAAATTAAGTGAAAAATA   Sequencing primer (5' to 3')     MT-co3   Exon   Forward primer (5' to 3')   TAATGATTTAAAAAATAACTCATAAAAATG     MT-co3   Exon   Forward primer (5' to 3')   GTATTATAAAAATCCAAAAAAATCC     MT-co3   Exon   Forward primer (5' to 3')   GTATTAAAAAATACTCAAAAAAATCC     L1-utr   -   Forward primer (5' to 3')   GTATTAAAAAATCCAAAAAAATCC     Sequencing primer (5' to 3')   GTATTAAAAATCTCAAAAAAATCC     Sequencing primer (5' to 3')   GTATTAAAAATCCAAAAAAATCC     Sequencing primer (5' to 3')   G			Sequencing primer (5' to 3')	
Int     Promoter     Forward primer (5' to 3')     GGATICITIATAGAATITIGGGGAGG       Reverse biotin primer (5' to 3')     ACTTCCTTAATAAAAAAACCATAATCTC       Sequence analyzed     C/TGTATTGGAGAAGAAATTGA       MT-co1     Exon     Forward primer (5' to 3')     AGTTGGAGTGGAATAGGAGAGAATA       Reverse biotin primer (5' to 3')     AGTTGGAGTGGAATAGAGAGAAATGA       MT-co1     Exon     Forward primer (5' to 3')     AGTTGGAGTGGAGTGGAGTGGAGGGGAATA       Reverse biotin primer (5' to 3')     TAAACTCCTAAATAAAAAACACCCCC     Sequence analyzed     C/TGGAAATTTAGGTGGGG/ATATTC/TGTAGATTTAA       MT-co2     Exon     Forward primer (5' to 3')     TAATGATTTAAAATTAGGTGAAATA     Sequencing primer (5' to 3')     TAATGATTTAAAATTAGGTGAATTT       MT-co3     Exon     Forward primer (5' to 3')     TAAGAATTTTAATTTTAGTTGAAGAC/TGTTTG       MT-co3     Exon     Forward primer (5' to 3')     AATAATAAAAAAATAACTCAAAAAAGGTT       MT-co3     Exon     Forward primer (5' to 3')     GTTATATAATTTAATTTTATTTTTTTTTTTTTTTGAGGT       Sequencing primer (5' to 3')     GTTATAAAAAAGGTTTT     Sequencing primer (5' to 3')     GATTAAAAAAGGTTTT       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGATAACCAAAAAATCCC     Sequencing primer (5' to 3')     GATAACCTGAAAAAAAACCCCC	- /	<b>a</b>	Sequence analyzed	C/IGATTITIA
MT-co1ExonForward primer (5' to 3')ACTTCCTTAATAAAAAACCATAATGAMT-co1ExonForward primer (5' to 3')TAAATTTTTGTTTTMT-co2ExonForward primer (5' to 3')AGTTGGAGATAGGATAGGATAGMT-co2ExonForward primer (5' to 3')TAACTCCTAAAATAAAAAACACCCCCSequencia primer (5' to 3')ATATTTTTTTTTTTAGTTSequencia primer (5' to 3')TAACTCCTAAATAAAAAACACCCCCSequencia primer (5' to 3')TAACTCCTAATATAAAAAAAACACCCCCMT-co2ExonForward primer (5' to 3')TAATGATTTAGTTTAGTTGGGG/ATATTC/TGTAGATTTAAMT-co3ExonForward primer (5' to 3')TAACCCTAATAAAAAAAAAAAATAACTCATAAATASequencia primer (5' to 3')ATAGAATTTTTAATTTSequencia primer (5' to 3')ATACCCTAATAAAAAAAAGGTTTMT-co3ExonForward primer (5' to 3')GTATAAAAAAAACTCCAAAAAAATCCSequencia primer (5' to 3')GTATAAAAAAAATACTCAAAAAAATCCSequencia primer (5' to 3')GTATAAAAAAAATACTCAAAAAAATCCSequencia primer (5' to 3')GTATAAAAAAATACTCAAAAAAATCCSequencia primer (5' to 3')GTATAAAAAAATACTCAAAAAAATCCSequencia primer (5' to 3')GTATAAAAAAACCCAACAACTTTCTGAAGAGTL1-utr-Forward primer (5' to 3')GTATAGAGGTTTTTGTTTGTTTGTTGTGTGTGTGTGTGT	Inf	Promoter	Forward primer (5' to 3')	GGAIIGIIAIAGAAIIIIGGIGAGG
MT-co1ExonSequence analyzed Forward primer (5' to 3')ITAAATITIGTTT CAGAAGAAATGAMT-co1ExonForward primer (5' to 3')AGTTGGAATGGAATGGAATA AGTTGGAATTAGGATGAATA Reverse biotin primer (5' to 3')TAACTCCTAAAATAAAAAACACCCCC Sequencing primer (5' to 3')MT-co2ExonForward primer (5' to 3')ATATTTTTTTTTTGTGGGG(ATTATGCGGAATTTAG Sequence analyzed C/TGGAAATTAAGTTAAGTTGAGGAATTT Reverse biotin primer (5' to 3')TAATGATTTAAGTTGGGG(ATATTC/TGTAGATTTAA MT-co2MT-co2ExonForward primer (5' to 3')TAATGATTTAGGTGAATTT Reverse biotin primer (5' to 3')TAACCCTAATAAAAAAATAACTCATAAATA Sequence analyzed C/TGTAATTAATTTTATTCTGAAGAC/TGTTTTGMT-co3ExonForward primer (5' to 3')ATAGAATTTTAATTTTTATTCTGAAGAC/TGTTTTG Sequence analyzed C/TGTAATTAATTATTTTTATTCTGAAGAC/TGTTTTGMT-co3ExonForward primer (5' to 3')GTTATTAATAAAAAAAAAAAAAACCC Sequencing primer (5' to 3')MT-co3ExonForward primer (5' to 3')GTTATTAATTTTTATTCTGAAGAC/TGTTTTG CAAAAAAATACTCAAAAAAATCC Sequence analyzed C/TGTAATAAAAAAAAAAAAACCCCAAAAAAATCC Sequence analyzed C/TGTAATAAAAAGGTTTL1-utr-Forward primer (5' to 3')GGTGTAATAGGATATTTGTTTGTG Reverse biotin primer (5' to 3')L1-utr-Forward primer (5' to 3')AAATTCACCAAACAACTTTCTTACAA Sequencing primer (5' to 3')L1-utr-Forward primer (5' to 3')AAATTCACCAAAAAAAAATAGTAAATTGGTGAGATTTTA Sequence analyzedL1-orf-Forward primer (5' to 3')AAATTCACCAAAAAATAGTAAAAAAAAAAAAAAAAAAAA			Reverse biotin primer (5' to 3')	
MT-co1ExonForward primer (5' to 3')AGTTGGAGGAGAAAATTGAMT-co1ExonForward primer (5' to 3')AGTTGGAGTTGGAATAGGATAAReverse biotin primer (5' to 3')ATATTTTTTTTTTAGTSequence analyzedC/TGGAAATTAAAAAAACACCCCMT-co2ExonForward primer (5' to 3')TAATGATTTAAAAAATAGGTGAATTMT-co3ExonForward primer (5' to 3')TAACCCTAATAAAAAAAATAACTCATAAATAMT-co3ExonForward primer (5' to 3')ATAGATTTTAAAAAAAAATAACTCATAAATAMT-co3ExonForward primer (5' to 3')GTTATTAATATTTTTATTGTATAAAAAGGTTMT-co3ExonForward primer (5' to 3')GTTATTAATAAAAAAAATACCCAAAAAAATCCMT-co3ExonForward primer (5' to 3')GTTATTAATATTTTTATTGTATAAAAAGGTTL1-utr-Forward primer (5' to 3')GTTATAAAAAAATACTCCAAAAAAATCCSequence analyzedC/TGATAC/TGGAATAATTTGTTTTC/TGAAGTL1-utr-Forward primer (5' to 3')GGTGTATAGGTTTTTGGTGGTGGTGL1-utr-Forward primer (5' to 3')AAATTAAAAAACACCAAAAATTC/TGTAAGAGTSequencing primer (5' to 3')GGTGTATAGGTTTTTTGGTGTGGTGGTGGTAGTATTTAL1-orf-Forward primer (5' to 3')AAATTCACCAAAACAACTTTCTAACAAL1-orf-Forward primer (5' to 3')AGAAGAATATTAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAGAATATTAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAGAATATTAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAGAATATTAAAAATAGTAAGGGAAAASequence analyzedC/TGTTGTAGAGAGTTC/TGTGGTAGTATTTA </td <td></td> <td></td> <td>Sequencing primer (5' to 3')</td> <td>TTAAATTTTTGTTTT</td>			Sequencing primer (5' to 3')	TTAAATTTTTGTTTT
MT-co1     Exon     Forward primer (5' to 3')     AGTTGGAATTAGGATGGATA       Reverse biotin primer (5' to 3')     TAACTCCTAAAATAAAAAAACACCCC     Reverse biotin primer (5' to 3')     TAACTCCTAAAATAAAAAAACACCCC       MT-co2     Exon     Forward primer (5' to 3')     TAATGATTTAGTTGGGG/ATATTC/TGTAGATTTAA       MT-co2     Exon     Forward primer (5' to 3')     TAATGATTTAAAATTAGGTGGAATTA       MT-co3     Exon     Forward primer (5' to 3')     TAACCCTAATAAAAAAAATAACTCATAAATA       Sequence analyzed     C/TGATATTTAATTTTATT     Sequence analyzed     C/TGATATTAAAAAAAAAAAATACTCAAAAAAGGTT       MT-co3     Exon     Forward primer (5' to 3')     GTTATTAATTTTATTGTATAAAAAAGGTT       MT-co3     Exon     Forward primer (5' to 3')     GTTATAAAAAAAGTTCCTGAAAAAAAGGTT       MT-co3     Exon     Forward primer (5' to 3')     GTTATAAAAAAAGTTTGTTATAAAATACCC       Sequence analyzed     C/TGATATTATTTTTTTTTTGTTTATTGTTTTC/TGAAGT     Sequencing primer (5' to 3')     AAATAAATAAAAATCCC       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGATTTTAGTTTTGTGTGG     Sequencing primer (5' to 3')     AAATCACCAAACAACTTTCTTACAA       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTTTGGTTGGTGGTG     Sequencing primer (5' to 3')     GGTGTATAGGTTTTTTGGTGTGG <td></td> <td></td> <td>Sequence analyzed</td> <td>C/TGTATTGGAGAAGAAATTGA</td>			Sequence analyzed	C/TGTATTGGAGAAGAAATTGA
MT-co2ExonReverse biotin primer (5' to 3')TAACTCCTAAAATTAAAAAACACCCCMT-co2ExonForward primer (5' to 3')ATATTTTTTTAGTTGGGG/ATATTC/TGTAGATTTAAMT-co3ExonForward primer (5' to 3')TAACCCCTAATAAAAAAAATAACTCATAAATAMT-co3ExonForward primer (5' to 3')ATAGATTTAATTTATTC/TGAAGAC/TGTTTGMT-co3ExonForward primer (5' to 3')ATAGATTTATTTTTATTC/TGAAGAC/TGTTTGMT-co3ExonForward primer (5' to 3')GTATAAAAAAAAAAAAAGGTTSequence analyzedC/TGTATATTTTATTGTAAAAAAGGTTMT-co3ExonForward primer (5' to 3')AAATAATAAAAAAGGTTTSequence analyzedC/TGTATAATTTTATTGTATAAAAAGGTTMT-co3ExonForward primer (5' to 3')GTATAAAAAGGTTTL1-utr-Forward primer (5' to 3')GTATAAAAAGGTTTTGGAGAAAAATCCSequencing primer (5' to 3')GGTGTATAC/TGGAATAATTTGTTTC/TGAAGTL1-utr-Forward primer (5' to 3')GGTGTATACGACAACAACTTTCTTACAASequencing primer (5' to 3')AAATTACCCAAACAACTTTCTTACAASequencing primer (5' to 3')GGTGTATAGGTTTTTGGTGTGGTAGTAGTTTTC/TGAAGTL1-utr-Forward primer (5' to 3')AAATTCACCAAACAACTTTCTACAASequencing primer (5' to 3')AAATTCACCAAACAACTTTTAACAASequencing primer (5' to 3')AAATTACCCAAAACAACTTTTACAAASequencing primer (5' to 3')AAATTAGGTGTGTGGTAGTATTTTAL1-orf-Forward primer (5' to 3')AGAAAGAATATTAAAAATGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAAGAATATTAAAAATGTAAGGGAAAAReverse biotin p	MT-co1	Exon	Forward primer (5' to 3')	AGTTGGAGTTGGAATAGGATGAATA
Sequencing primer (5' to 3')ATATTITITITAGT Sequence analyzedMT-co2ExonForward primer (5' to 3')TAATGATTTAAATTAGGTGAATTT Neverse biotin primer (5' to 3')MT-co3ExonForward primer (5' to 3')TAATGATTTAAAATAACTCATAAATA Sequence analyzedMT-co3ExonForward primer (5' to 3')ATAGAATTTTATTTTATTC/TGAAGAC/TGTTTTG Sequence analyzedMT-co3ExonForward primer (5' to 3')GTTATTAATATTTTTTTTTTTTTTTGTTAAAAAAGGTT Sequence analyzedMT-co3ExonForward primer (5' to 3')GTTATTAAAAAAAAAACGTTTT Sequence analyzedL1-utr-Forward primer (5' to 3')GTATTAAAAAAGGTTT GTATAAAAAGGTTTTTGTTGTTGTGTG Sequencing primer (5' to 3')L1-utr-Forward primer (5' to 3')GTGTATAGGATTATTTGTTGTGTG GTGTATAGGTTTTTTGTTGTTGTGTGL1-orf-Forward primer (5' to 3')AAATCACCAAACAACTTTCTAAAAAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3')ATCAATCCAAAATATTAAAAATAGTAAGGGAAAA AAATCACCAAAATATTAAAAATAGTAAGGGAAAAA Reverse biotin primer (5' to 3')L1-orf-Forward primer (5' to 3')ATCAATCCAAAATATTAAAAATAGTAAGGGAAAAA Reverse biotin primer (5' to 3')L1-orf-Forward primer (5' to 3')ATCAATCCAAAATATTAAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3')L1-orf-Forward primer (5' to 3')ATCAATCCAAAATTTAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3')L1-orf-Forward primer (5' to 3')ATCAATCCAAAATTTAAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3')L1-orf-Forward primer (5' to 3')ATCAATCCAAAATTTAAAAATAGTAAGGGAAAA Reverse biotin pr			Reverse biotin primer (5' to 3')	TAACTCCTAAAATAAAAAACACCCC
MT-co2ExonSequence analyzedC/TGGAAATTTAGTTTAGTTGGGG/ATATTC/TGTAGATTTAAMT-co2ExonForward primer (5' to 3')TAATGATTTAAAATTAGGTGAATTTReverse biotin primer (5' to 3')ATAGAATTTTAATTTAAAATTAGCTCATAAAATASequencing primer (5' to 3')ATAGAATTTTATTTATTMT-co3ExonForward primer (5' to 3')GTTATTATTATTTTTTTTTTTTTGTAAAAATAACCCMT-co3ExonForward primer (5' to 3')GTTATTAATTTTTTTTTTTTTTTTTTTGTTAAAAATACCCMT-co3ExonForward primer (5' to 3')GTTATTAAAAAAAAAAACCCL1-utr-Forward primer (5' to 3')GTGTATACTGGAAATAATTTGTTTTC/TGAAGTL1-utr-Forward primer (5' to 3')GTGTATAGGTTTTTTGGTTGTTGL1-orf-Forward primer (5' to 3')GTGTATGGAGAGTTC/TGTGGTAGTATTTTAL1-orf-Forward primer (5' to 3')AAATCACCAAAAAATGTAAAAAAGGGGAAAAReverse biotin primer (5' to 3')AGAAAGAATATTAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAAGAATATTAAAAATAGTAAGGGAAAAL1-orf-Forward primer (5' to 3')ATCAATCCAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAAGAATATTAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')ATCAATCCAAAATAGTAAGGAAAATAGTAAGGGAAAAL1-orf-Forward primer (5' to 3')ATCAATCCAAAATAGTAAGGAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')ATCAATCCAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')Reverse biotin primer (5' to 3')ATCAATCCAAAATAGTAAGGAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')Reverse biotin primer (5' to 3') <td< td=""><td></td><td></td><td>Sequencing primer (5' to 3')</td><td>ATATTTTTTTAGT</td></td<>			Sequencing primer (5' to 3')	ATATTTTTTTAGT
MT-co2ExonForward primer (5' to 3')TAATGATTTAAAATTAGGTGAATTT Reverse biotin primer (5' to 3')MT-co3ExonForward primer (5' to 3')ATAGAATTTTAATTT Sequence analyzedMT-co3ExonForward primer (5' to 3')GTTATTAATTTTATTC/TGAAGAC/TGTTTTGMT-co3ExonForward primer (5' to 3')GTTATTAATTTTATTTTATTGTATAAAAAGGTT Reverse biotin primer (5' to 3')L1-utr-Forward primer (5' to 3')GTTATAAATAAAAATACTCAAAAAAATCC Sequencing primer (5' to 3')L1-utr-Forward primer (5' to 3')GTTATAAATTTTGTTTATTGTTTATTGTTTC/TGAAGT GGTGTATAGGTTTTTTGGTTGTTG Reverse biotin primer (5' to 3')L1-utr-Forward primer (5' to 3')GGTGTATAGGTTTTTTGGTTGTG GGTGTATAGGTTTTTTGGTTGTTGL1-orf-Forward primer (5' to 3')AAATAAAATGAGAGAGTTC/TGTGGTAGTATTTTA Sequence analyzedL1-orf-Forward primer (5' to 3')AGAAAGAATATTAAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3')L1-orf-Forward primer (5' to 3')AGAAAGAATATTAAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3')L1-orf-Forward primer (5' to 3')AGAAAGAATATTAAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3')L1-orf-Forward primer (5' to 3')AGAAAGAATATTAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3')L1-orf-Forward primer (5' to 3')AGAAAGAATATTAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3')CTCTTACAAAATTTAAATTAAGTTTTTSequencing primer (5' to 3')ATTAATTTAAATTAAGTTAAGGGAAAA Reverse biotin primer (5' to 3')			Sequence analyzed	C/TGGAAATTTAGTTTATGTTGGGGG/ATATTC/TGTAGATTTAA
Reverse biotin primer (5' to 3')TAACCCTAATAAAAAATAACTCATAAATASequencing primer (5' to 3')ATAGAATTTTAATTSequence analyzedC/TGTATATTATTTTATTC/TGAAGAC/TGTTTGMT-co3ExonForward primer (5' to 3')GTATTAAAAAAAAAAAAACCCReverse biotin primer (5' to 3')AAATAATAAAAAACCCAAAAAAATCCSequence analyzedC/TGATACTTGAAAAAAAACCCL1-utr-Forward primer (5' to 3')GTATAAAAAGGTTTTGTTATGTTATGTTATC/TGAAGACL1-utr-Forward primer (5' to 3')GGTGTATAGGTTTTTGGTTGTGTL1-orf-Forward primer (5' to 3')AAATTCACCAAACAACTTCTTACAAL1-orf-Forward primer (5' to 3')AGAAAGAATATTAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAAGAATATTAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AAATTCACCCAAACAACTTTCTTACAAL1-orf-Forward primer (5' to 3')AGAAAGAATATTAAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAAGAATATTAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAAGAATATTAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')AGAAAGAATATTAAAATAGTAAGGGAAAAReverse biotin primer (5' to 3')ATCATTCAAAATTAAGTAAGGGAAAAReverse biotin primer (5' to 3')ATCAATTCAAATTAAGTAAGGGAAAAReverse biotin primer (5' to 3')TTATT	MT-co2	Exon	Forward primer (5' to 3')	TAATGATTTAAAATTAGGTGAATTT
MT-co3Sequencing primer (5' to 3')ATAGAATTTTAATTMT-co3ExonForward primer (5' to 3')GTTATTAATTTATTC/TGAAGAC/TGTTTGReverse biotin primer (5' to 3')GTTATTAAAAAAAAAAAAAAACCCSequencing primer (5' to 3')AAATAAAAAAGGTTTSequencing primer (5' to 3')GTATAAAAAAGGTTTATTGTTTATTGTTATGTTATGTT			Reverse biotin primer (5' to 3')	ΤΑΑϹϹϹΤΑΑΤΑΑΑΑΑΑΑΤΑΑϹΤϹΑΤΑΑΑΤΑ
MT-co3     Exon     Forward primer (5' to 3')     GTTATTAATTTTATTC/TGAAGAC/TGTTTTG       MT-co3     Exon     Forward primer (5' to 3')     GTTATTATATTTTATTGTATAAAAAGGTT       Reverse biotin primer (5' to 3')     AAATAATAAAAATACTCAAAAAAATCC     Sequencing primer (5' to 3')     AAATAATAAAAAGGTTTT       L1-utr     -     Forward primer (5' to 3')     GTGTATAGGTATTGTTTATTGTTTATTGTTTC/TGAAGT       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTGGTTGTTG       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTGGTTGTTG       L1-utr     -     Forward primer (5' to 3')     AAATTCACCAAACAACTTTCTTACAA       Sequencing primer (5' to 3')     GGTGTATAGGTTGTTG     Sequencing primer (5' to 3')       L1-orf     -     Forward primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       L1-orf     -     Forward primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA     Sequencing primer (5' to 3')     ATCAATCCAAAATCTTCAACCTTC       Sequencing primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA     Sequencing primer (5' to 3')     ATCAATCCAAATTTAACTTAACCTTC       Sequencing primer (5' to 3')     TTATATAGATTTTT     Sequencing primer (5' to 3')     ATCAATCCAAATTTAACTTAAC			Sequencing primer (5' to 3')	ATAGAATTTTTAATT
MT-co3     Exon     Forward primer (5' to 3')     GTTATTATATTTTATTGTATAAAAAGGTT       Reverse biotin primer (5' to 3')     AAATAATAAAAATACTCAAAAAAATCC     Sequencing primer (5' to 3')     AAATAATAAAAAGGTTT       L1-utr     -     Forward primer (5' to 3')     GTATTAAAAAGGTTTGTTTGTTTATTGTTTC/TGAAGT       L1-utr     -     Forward primer (5' to 3')     GTGTATAGGTTTTTGTTTGTTGTTGTGTG       Reverse biotin primer (5' to 3')     GGTGTATAGGTTTTTGGTTGTTGGTAGTAGT       L1-utr     -     Forward primer (5' to 3')     AAATTCACCAAACAACTTTCTACAA       L1-utr     -     Forward primer (5' to 3')     AAATTCACCAAACAACTTTACAA       L1-orf     -     Forward primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       L1-orf     -     Forward primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     ATCAATCCAAAATCTTCTAACCTTC       Sequencing primer (5' to 3')     ATCAATCCAAATTTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     ATCAATCCAAATTTAACTTAACCTTC       Sequencing primer (5' to 3')     ATCAATCCAAATTTAACCTTC       Sequencing primer (5' to 3')     ATCAATCCAAATTTAACCTTC       Sequencing primer (5' to 3')     TTATTACAAATTTAAC			Sequence analyzed	C/TGTATATTAATTTTATTC/TGAAGAC/TGTTTTG
Reverse biotin primer (5' to 3')     AAATAATAAAATACTCAAAAAAATCC       Sequencing primer (5' to 3')     GTATAAAAAGGTTTT       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTTGTTTATTGTTTATTGTTTC/TGAAGT       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTTTGGTTGTTG       L1-utr     -     Forward primer (5' to 3')     AAATTCACCAAACAACTTTCTTACAA       Sequencing primer (5' to 3')     AAATTCACCCAAACAACTTTCTTACAA     Sequencing primer (5' to 3')       L1-orf     -     Forward primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       L1-orf     -     Forward primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA     Reverse biotin primer (5' to 3')       L1-orf     -     Forward primer (5' to 3')     ATTATAGAATTTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     TTATATTAGATTTTT       Sequencing primer (5' to 3')     TTATATTAGATTTTTAACTTCAACCTTC       Sequencing primer (5' to 3')     TTATATTAGATTTAACCTTAACTTAACTTAACTTAACTT	MT-co3	Exon	Forward primer (5' to 3')	GTTATTATATTTTATTGTATAAAAAGGTT
L1-utr     -     Forward primer (5' to 3')     GTATAAAAAGGTTTT       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTTGTTTATTGTTTATTGTTTC/TGAAGT       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTTTGGTTGTTG       L1-utr     -     Forward primer (5' to 3')     AAATTCACCAAACAACTTTCTTACAA       Sequencing primer (5' to 3')     AAATTCACCAAACAACTTTCTTACAA     Sequencing primer (5' to 3')       L1-orf     -     Forward primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     ATCAATCCAAAATCTTCAACCTTC       Sequencing primer (5' to 3')     TTATATAGATTTTT       Sequencing primer (5' to 3')     TTATATTAGATTTTT			Reverse biotin primer (5' to 3')	ΑΑΑΤΑΑΤΑΑΑΑΤΑCΤCAAAAAATCC
L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTTGTTTGTTGTTG       L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTTTGGTTGTTG       Reverse biotin primer (5' to 3')     AAATTCACCAAACAACTTTCTTACAA     Sequencing primer (5' to 3')       L1-orf     -     Forward primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     ATCAATCCAAAATCTTCAACCTTC       Sequencing primer (5' to 3')     TTATATTAGATTTTT       CTTGTTACAAAATTAT     Sequencing primer (5' to 3')			Sequencing primer (5' to 3')	GTATAAAAAGGTTTT
L1-utr     -     Forward primer (5' to 3')     GGTGTATAGGTTTTTTGGTTGTTG       Reverse biotin primer (5' to 3')     AAATTCACCAAACAACTTTCTTACAA       Sequencing primer (5' to 3')     TTTTTTGGTTGTTGT       Sequence analyzed     C/TGTTGTAGAGAGTTC/TGTGGTAGTATTTTA       L1-orf     -     Forward primer (5' to 3')       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGCAATTTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     ATCAATCCAAAATCTTCAACCTTC       Sequencing primer (5' to 3')     TTATATTAGATTTTT       Sequencing primer (5' to 3')     GTGTATAGATATTAA			Sequence analyzed	C/TGATAC/TGGAATAATTTTGTTTATTGTTTC/TGAAGT
Reverse biotin primer (5' to 3')     AAATTCACCAAACAACTTTCTTACAA       Sequencing primer (5' to 3')     TTTTTTGGTTGTTGT       Sequence analyzed     C/TGTTGTAGAGAGTTC/TGTGGTAGTATTTTA       L1-orf     -     Forward primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     AGAAAGAATATTAAAAATAGTAAGGGAAAA       Reverse biotin primer (5' to 3')     ATCAATCCAAAATCTTCTAACCTTC       Sequencing primer (5' to 3')     TTATATTAGATTTTT	L1-utr	-	Forward primer (5' to 3')	GGTGTATAGGTTTTTTGGTTGTTG
L1-orf - Forward primer (5' to 3') TTTTTTGGTTGTTGT Forward primer (5' to 3') AGAAGAATATTAAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3') AGAAGAATATTAAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3') ATCAATCCAAAATCTTCTAACCTTC Sequencing primer (5' to 3') TTATATTAGATTTTT			Reverse biotin primer (5' to 3')	ΑΑΑΤΤΟΑΟΟΑΑΑΟΑΑΟΤΤΤΟΤΤΑΟΑΑ
L1-orf - Forward primer (5' to 3') AGAAAGAATATTAAAAATAGTAAGGGAAAA Reverse biotin primer (5' to 3') ATCAATCCAAAATCTTCAACCTTC Sequencing primer (5' to 3') TTATATTAGAATTTT Sequencing primer (5' to 3') CCCTTACAAAATTAT			Sequencing primer (5' to 3')	TTTTTGGTTGTTGT
L1-orf - Forward primer (5' to 3') AGAAAGAATATTAAAAATAGTAAGGAAAA Reverse biotin primer (5' to 3') ATCAATCCTAAACTTCTAAACTTC Sequencing primer (5' to 3') TTATATTAGATTTTT Sequences pailward			Sequence analyzed	C/TGTTGTAGAGAGTTC/TGTGGTAGTATTTTA
Reverse biotin primer (5' to 3') Sequencing primer (5' to 3') ATCAATCCAAAATCTTCTAACCTTC Sequencing primer (5' to 3') ATCATTAGATTTTT CTTCTTAGATATTAT	L1-orf	-	Forward primer (5' to 3')	AGAAAGAATATTAAAAATAGTAAGGAAAA
Sequencing primer (5' to 3') TTATATTAGATTTTT			Reverse biotin primer (5' to 3')	ΑΤCΑΑΤCCAAAATCTTCTAACCTTC
Sequence applicate Contract Addition			Sequencing primer (5' to 3')	TTATATTAGATTITT
			Sequence analyzed	C/TGTTAGAAATTAT

manufacturer's instructions. Purified DNA was stored at Acknowledgments  $-20^{\circ}$ C until analysis.

#### **DNA** methylation analysis

Gene-specific and global 5mC DNA methylation were analyzed by pyrosequencing. Global methylation levels were estimated using two assays to interrogate LINE1 methylation (L1-utr and L1-orf) that have been widely utilized for this purpose since their first development [59] and which correlate well with measures of global 5mC content by high-performance liquid chromatography [60]. Gene-specific assays were designed to interrogate promoter regions for the Bdnf, Comt, Il1b, Il6, Nr3c1, and Tnf genes. Specifically, the assays interrogated Bdnf promoter IV, which influences response to levomethadone [15]; the *Comt* P1 promoter, which regulates expression of the shorter tissue-specific S-COMT isoform, which influences dopamine metabolism in the mouse brain [61]; and the Nr3c1 GR110 promoter, which we have previously demonstrated to be differentially methylated in the rat brain in response to environmental exposures [62].

Bisulfite conversion was performed using 1 µg of genomic DNA and the EZ-96 DNA Methylation-Gold Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. M-Elution Buffer (30 µl) was used for the elution of bisulfite-converted DNA. Following amplification of target regions by PCR, DNA methylation was analyzed by pyrosequencing. Details of the primers and thermocycling conditions are shown in Table 3. In brief, a 30 µl-PCR was carried out using 15 µl GoTaq Hot Start Green Master Mix (Promega), 10 pmol forward primer, 10 pmol reverse primer, 1 µl bisulfite-treated DNA, and water. Pyrosequencing was performed using the PyroMark Q96 MD Pyrosequencing System (QIAGEN, Germantown, MD). The percentage of methylated cytosines was quantified at three CpG sites for Bdnf, two for Comt, two for Il1b, one for Il6, six for Nr3c1, one for Tnf, two for L1-utr, and one for L1-orf. For the mitochondrially-encoded genes, the percentage-methylation was measured at three CpG sites for each of Mtco1, Mtco2, and Mtco3. Pyrosequencing reactions were performed in duplicate and the mean of the replicates taken forward for analysis. The correlation coefficient between replicate pyrosequencing runs ranged from 0.77 (L1-orf) to 0.97 (Comt).

#### Analysis of global 5-hydroxymethylcytosine levels

The 5hmC modification was measured in total DNA using the MethylFlash Global DNA Hydroxymethylation ELISA Easy Kit (Epigentek, Farmingdale, NY) according to the manufacturer's protocol. The correlation coefficient between replicates was 0.97.

#### **Statistical analysis**

Analysis was performed using GraphPad Prism version 7.0b. Differences in DNA methylation were determined by t-test, with significance defined as P value < 0.05.

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#### **Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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#### References

- [1] Hutchinson MR, Shavit Y, Grace PM, et al. Exploring the neuroimmunopharmacology of opioids: an integrative review of mechanisms of central immune signaling and their implications for opioid analgesia. Pharmacol Rev. 2011;63:772-810; PMID:21752874; Available from: https://doi.org/10.1124/pr.110.004135
- [2] Albertson DN, Schmidt CJ, Kapatos G, et al. Distinctive profiles of gene expression in the human nucleus accumbens associated with cocaine and heroin abuse. Neuropsychopharmacology. 2006;31:2304-2312; PMID:16710320; Available from: https://doi. org/10.1038/sj.npp.1301089
- Piechota M, Korostynski M, Sikora M, et al. Common transcriptional [3] effects in the mouse striatum following chronic treatment with heroin and methamphetamine. Genes Brain Behav. 2012;11:404-414; PMID:22390687; Available from: https://doi.org/10.1111/j.1601-183X.2012.00777.x
- [4] Hutchinson MR, Coats BD, Lewis SS, et al. Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. Brain Behav Immun. 2008;22:1178-1189; PMID:18599265; Available from: https://doi.org/10.1016/j.bbi.2008.05.004
- [5] Shavit Y, Wolf G, Goshen I, et al. Interleukin-1 antagonizes morphine analgesia and underlies morphine tolerance. Pain. 2005;115:50-59; PMID:15836969; Available from: https://doi.org/ 10.1016/j.pain.2005.02.003
- [6] Vargas-Perez H, Ting-A Kee R, Walton CH, et al. Ventral tegmental area BDNF induces an opiate-dependent-like reward state in naive rats. Science. 2009;324:1732-1734; PMID:19478142; Available from: https://doi.org/10.1126/science.1168501
- Matsushita Y, Omotuyi IO, Mukae T, et al. Microglia activation pre-[7] cedes the anti-opioid BDNF and NMDA receptor mechanisms underlying morphine analgesic tolerance. Curr Pharm Des. 2013;19:7355-7361; PMID:23448475; Available from: https://doi. org/10.2174/138161281942140105161733
- [8] Kambur O, Männistö PT, Viljakka K, et al. Stress-induced analgesia and morphine responses are changed in catechol-O-methyltransferase-deficient male mice. Basic Clin Pharmacol Toxicol. 2008;103:367-373; PMID:18834357; Available from: https://doi.org/ 10.1111/j.1742-7843.2008.00289.x
- [9] Zhu J, Zhu F, Zhao N, et al. Methylation of glucocorticoid receptor gene promoter modulates morphine dependence and accompanied hypothalamus-pituitary-adrenal axis dysfunction. J Neurosci Res. 2017;95:1459-1473; PMID:27618384; Available from: https://doi. org/10.1002/jnr.23913

- [10] Cunha-Oliveira T, Rego AC, Garrido J, et al. Street heroin induces mitochondrial dysfunction and apoptosis in rat cortical neurons. J Neurochem. 2007;101:543–554; PMID:17250679; Available from: https://doi.org/10.1111/j.1471-4159.2006.04406.x
- [11] Mastronicola D, Arcuri E, Arese M, et al. Morphine but not fentanyl and methadone affects mitochondrial membrane potential by inducing nitric oxide release in glioma cells. Cell Mol Life Sci. 2004;61:2991–2997; PMID:15583861; Available from: https://doi. org/10.1007/s00018-004-4371-x
- [12] Feng YM, Jia YF, Su LY, et al. Decreased mitochondrial DNA copy number in the hippocampus and peripheral blood during opiate addiction is mediated by autophagy and can be salvaged by melatonin. Autophagy. 2013;9:1395–1406; PMID:23800874; Available from: https://doi.org/10.4161/auto.25468
- Skrabalova J, Drastichova Z, Novotny J. Morphine as a potential oxidative stress-causing agent. Mini Rev Org Chem. 2013;10:367–372; PMID:24376392; Available from: https://doi.org/10.2174/ 1570193X113106660031
- Sadakierska-Chudy A, Frankowska M, Filip M. Mitoepigenetics and drug addiction. Pharmacol Ther. 2014;144:226–233; PMID:24956109; Available from: https://doi.org/10.1016/j.pharm thera.2014.06.002
- [15] Schuster R, Kleimann A, Rehme M-K, et al. Elevated methylation and decreased serum concentrations of BDNF in patients in levomethadone compared to diamorphine maintenance treatment. Eur Arch Psychiatry Clin Neurosci. 2017;267:33–40; PMID:26801497; Available from: https://doi.org/10.1007/s00406-016-0668-7
- [16] Xu X, Ji H, Liu G, et al. A significant association between *BDNF* promoter methylation and the risk of drug addiction. Gene. 2016;584:54–59; PMID:26976342; Available from: https://doi.org/ 10.1016/j.gene.2016.03.010
- [17] Marie-Claire C, Crettol S, Cagnard N, et al. Variability of response to methadone: genome-wide DNA methylation analysis in two independent cohorts. Epigenomics. 2016;8:181–195; PMID:26792095; Available from: https://doi.org/10.2217/epi.15.110
- [18] Nielsen DA, Yuferov V, Hamon S, et al. Increased OPRM1 DNA methylation in lymphocytes of methadone-maintained former heroin addicts. Neuropsychopharmacology. 2009;34:867–873; PMID:18650805; Available from: https://doi.org/10.1038/npp.2008. 108
- [19] Chao M-R, Fragou D, Zanos P, et al. Epigenetically modified nucleotides in chronic heroin and cocaine treated mice. Toxicol Lett. 2014;229:451–457; PMID:25064621; Available from: https://doi.org/ 10.1016/j.toxlet.2014.07.023
- [20] Fragou D, Zanos P, Kouidou S, et al. Effect of chronic heroin and cocaine administration on global DNA methylation in brain and liver. Toxicol Lett. 2013;218:260–265; PMID:23454526; Available from: https://doi.org/10.1016/j.toxlet.2013.01.022
- [21] McFalls AJ, Imperio CG, Bixler G, et al. Reward devaluation and heroin escalation is associated with differential expression of CRF signaling genes. Brain Res Bull. 2016;123:81–93; PMID:26655889; Available from: https://doi.org/10.1016/j.brainresbull.2015.11.009
- [22] Kozlenkov A, Jaffe AE, Timashpolsky A, et al. DNA Methylation profiling of human prefrontal cortex neurons in heroin users shows significant difference between genomic contexts of hyper- and hypomethylation and a younger epigenetic age. Genes (Basel). 2017;PMID:828556790; Available from: https://doi.org/10.3390/genes8060152
- [23] Bolaños CA, Nestler EJ. Neurotrophic mechanisms in drug addiction. Neuromolecular Med. 2004;5:69–83; PMID:15001814; Available from: https://doi.org/10.1385/NMM:5:1:069
- [24] Mashayekhi FJ, Rasti M, Rahvar M, et al. Expression levels of the BDNF gene and histone modifications around its promoters in the ventral tegmental area and locus ceruleus of rats during forced abstinence from morphine. Neurochem Res. 2012;37:1517–1523; PMID:22410736; Available from: https://doi.org/10.1007/s11064-012-0746-9
- [25] Commons KG. Neuronal pathways linking substance P to drug addiction and stress. Brain Res. 2010;1314:175–182; PMID:19913520; Available from: https://doi.org/10.1016/j.brainres.2009.11.014
- [26] De Ross J, Avila MA, Ruggiero RN, et al. The unconditioned fear produced by morphine withdrawal is regulated by mu- and kappa-

opioid receptors in the midbrain tectum. Behav Brain Res. 2009;204:140–146; PMID:19520121; Available from: https://doi.org/ 10.1016/j.bbr.2009.05.033

- [27] Mihaly E, Legradi G, Fekete C, et al. Efferent projections of ProTRH neurons in the ventrolateral periaqueductal gray. Brain Res. 2001;919:185–197; PMID:11701131; Available from: https://doi.org/ 10.1016/S0006-8993(01)02962-6
- [28] Alvandi MS, Bourmpoula M, Homberg JR, et al. Association of contextual cues with morphine reward increases neural and synaptic plasticity in the ventral hippocampus of rats. Addict Biol. 2017;919:185–197; PMID:28940732; Available from: https://doi.org/ 10.1111/adb.12547
- [29] Fuchs RA, Evans KA, Ledford CC, et al. The role of the dorsomedial prefrontal cortex, basolateral amygdala, and dorsal hippocampus in contextual reinstatement of cocaine seeking in rats. Neuropsychopharmacology. 2005;30:296–309; PMID:15483559; Available from: https://doi.org/10.1038/sj.npp.1300579
- [30] Taylor JA, Ivry RB. Cerebellar and prefrontal cortex contributions to adaptation, strategies, and reinforcement learning. Prog Brain Res. 2014;210:217–253; PMID:24916295; Available from: https://doi.org/ 10.1016/B978-0-444-63356-9.00009-1
- [31] Vera-Portocarrero LP, Ossipov MH, Lai J, et al. Descending facilitatory pathways from the rostroventromedial medulla mediate naloxone-precipitated withdrawal in morphine-dependent rats. J Pain. 2011;12:667–676; PMID:21354865; Available from: https://doi.org/ 10.1016/j.jpain.2010.12.007
- [32] Bora E, Yücel M, Fornito A, et al. White matter microstructure in opiate addiction. Addict Biol. 2012;17:141–148; PMID:21070508; Available from: https://doi.org/10.1111/j.1369-1600.2010.00266.x
- [33] Zhu Y, Wienecke CF, Nachtrab G, et al. A thalamic input to the nucleus accumbens mediates opiate dependence. Nature. 2016;530:219–222; PMID:26840481; Available from: https://doi.org/ 10.1038/nature16954
- [34] Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science. 2009;324:929–930; PMID:19372393; Available from: https://doi.org/ 10.1126/science.1169786
- [35] Song C-X, Szulwach KE, Fu Y, et al. Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nat Biotechnol. 2011;29:68–72; PMID:21151123; Available from: https://doi.org/10.1038/nbt.1732
- [36] Nestor CE, Ottaviano R, Reddington J, et al. Tissue type is a major modifier of the 5-hydroxymethylcytosine content of human genes. Genome Res. 2012;22:467–477; PMID:22106369; Available from: https://doi.org/10.1101/gr.126417.111
- [37] Khare T, Pai S, Koncevicius K, et al. 5-hmC in the brain is abundant in synaptic genes and shows differences at the exon-intron boundary. Nat Struct Mol Biol. 2012;19:1037–1043; PMID:22961382; Available from: https://doi.org/10.1038/nsmb.2372
- [38] Jin S-G, Kadam S, Pfeifer GP. Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5hydroxymethylcytosine. Nucleic Acids Res. 2010;38:e125; PMID:20371518; Available from: https://doi.org/10.1093/nar/gkq223
- [39] Feng J, Shao N, Szulwach KE, et al. Role of Tet1 and 5-hydroxymethylcytosine in cocaine action. Nat Neurosci. 2015;18:536– 544; PMID:25774451; Available from: https://doi.org/10.1038/ nn.3976
- [40] Sadakierska-Chudy A, Frankowska M, Wydra K, et al. Increased 5hydroxymethylation levels in the hippocampus of rat extinguished from cocaine self-administration. Hippocampus. 2017;27:811–821; PMID:28422379; Available from: https://doi.org/10.1002/hipo.22733
- [41] Ge XQ, Xu PC, Bian CF. Relationship between morphine-induced respiratory depression and the cholinergic system of respiratory center. Yao Xue Xue Bao. 1990;25:566–572; 2082678
- [42] Akbarian S, Rios M, Liu R-J, et al. Brain-derived neurotrophic factor is essential for opiate-induced plasticity of noradrenergic neurons. J Neurosci. 2002;22:4153–4162; PMID:12019333; Available from: https://doi.org/20026381
- [43] Laviolette SR, van der Kooy D. GABA(A) receptors in the ventral tegmental area control bidirectional reward signalling between

dopaminergic and non-dopaminergic neural motivational systems. Eur J Neurosci. 2001;13:1009–1015; PMID:11264674; Available from: https://doi.org/10.1046/j.1460-9568.2001.01458.x

- [44] Rakvåg TT, Klepstad P, Baar C, et al. The Val158Met polymorphism of the human catechol-O-methyltransferase (COMT) gene may influence morphine requirements in cancer pain patients. Pain. 2005;116:73–78; PMID:15927391; Available from: https://doi.org/ 10.1016/j.pain.2005.03.032
- [45] Ninković J, Roy S. Role of the mu-opioid receptor in opioid modulation of immune function. Amino Acids. 2013;45:9–24; PMID:22170499; Available from: https://doi.org/10.1007/s00726-011-1163-0
- [46] Houghtling RA, Bayer BM. Rapid elevation of plasma interleukin-6 by morphine is dependent on autonomic stimulation of adrenal gland. J Pharmacol Exp Ther. 2002;300:213–219; PMID:11752119; Available from: https://doi.org/10.1124/jpet.300.1.213
- [47] Zubelewicz B, Muc-Wierzgoń M, Harbuz MS, et al. Central single and chronic administration of morphine stimulates corticosterone and interleukin (IL)-6 in adjuvant-induced arthritis. J Physiol Pharmacol. 2000;51:897–906; PMID:11220497.
- [48] Roy S, Cain KJ, Chapin RB, et al. Morphine modulates NF kappa B activation in macrophages. Biochem Biophys Res Commun. 1998;245:392–396; PMID:9571161; Available from: https://doi.org/ 10.1006/bbrc.1998.8415
- [49] Enrico P, Esposito G, Mura MA, et al. Effects of allopurinol on striatal dopamine, ascorbate and uric acid during an acute morphine challenge: ex vivo and in vivo studies. Pharmacol Res. 1997;35:577– 585; PMID:9356212; Available from: https://doi.org/10.1006/ phrs.1997.0193
- [50] Wu Q, Ni X. ROS-mediated DNA methylation pattern alterations in carcinogenesis. Curr Drug Targets. 2015;16:13–19; PMID:25585126; Available from: https://doi.org/10.2174/1389450116666150113121054
- [51] Baccarelli AA, Byun H-M. Platelet mitochondrial DNA methylation: a potential new marker of cardiovascular disease. Clin Epigenetics. 2015;7:44; PMID:25901189; Available from: https://doi.org/10.1186/ s13148-015-0078-0
- [52] Byun H-M, Panni T, Motta V, et al. Effects of airborne pollutants on mitochondrial DNA methylation. Part Fibre Toxicol. 2013;10:18; PMID:23656717; Available from: https://doi.org/10.1186/1743-8977-10-18
- [53] Byun H-M, Colicino E, Trevisi L, et al. Effects of air pollution and blood mitochondrial DNA methylation on markers of heart rate

variability. J Am Heart Assoc. 2016;5:e003218; PMID:27107129; Available from: https://doi.org/10.1161/JAHA.116.003218

- [54] Middleton J, McGrail S, Stringer K. Drug related deaths in England and Wales. BMJ. 2016;355:i5259; PMID:27754839; Available from: https://doi.org/10.1136/bmj.i5259
- [55] Rudd RA, Seth P, David F, et al. Increases in Drug and Opioid-Involved Overdose Deaths – United States, 2010–2015. MMWR Morb Mortal Wkly Rep. 2016;65:1445–1452; PMID:28033313; Available from: https://doi.org/10.15585/mmwr.mm655051e1
- [56] Trujillo KA, Akil H. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. Science. 1991;251:85–87; PMID:1824728; Available from: https://doi.org/ 10.1126/science.1824728
- [57] Chao YC, Xie F, Li X, et al. Demethylation regulation of BDNF gene expression in dorsal root ganglion neurons is implicated in opioidinduced pain hypersensitivity in rats. Neurochem Int. 2016;97:91–98; PMID:26970395; Available from: https://doi.org/10.1016/j.neu int.2016.03.007
- [58] Sun H, Maze I, Dietz DM, et al. Morphine epigenomically regulates behavior through alterations in histone H3 lysine 9 dimethylation in the nucleus accumbens. J Neurosci. 2012;32:17454–17464; PMID:23197736; Available from: https://doi.org/10.1523/JNEURO SCI.1357-12.2012
- [59] Yang AS, Estécio MR, Doshi K, et al. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res. 2004;32:38e; PMID:14973332; Available from: https://doi.org/10.1093/nar/gnh032
- [60] Lisanti S, Omar WA, Tomaszewski B, et al. Comparison of methods for quantification of global DNA methylation in human cells and tissues. PLoS One. 2013;8:e79044; PMID:24260150; Available from: https://doi.org/10.1371/journal.pone.0079044
- [61] Tammimäki A, Käenmäki M, Kambur O, et al. Effect of S-COMT deficiency on behavior and extracellular brain dopamine concentrations in mice. Psychopharmacology (Berl). 2010;211:389–401; PMID:20617305; Available from: https://doi.org/10.1007/s00213-010-1944-2
- [62] Byun HM, Benachour N, Zalko D, et al. Epigenetic effects of low perinatal doses of flame retardant BDE-47 on mitochondrial and nuclear genes in rat offspring. Toxicology. 2015;328:152–159; PMID:25533936; Available from: https://doi.org/10.1016/j.tox.2014.12.019